Exploring the energetic and behavioural changes associated with the immune response in zebrafish (*Danio rerio*)

by

Nathan R. Bennoit

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

With the world's population continuously increasing, humanity constantly needs to figure out ways to increase food production to meet growing demands. One way to increase food production that has had exponential growth in recent years is aquaculture. This technique of cultivating fish has allowed society to meet protein needs; however, it has brought some challenges as well. One challenge is the management of disease that affects fish in aquaculture because of the high stocking densities and in some case interactions with an unfamiliar environment. The high prevalence of disease impacts the profits of the aquaculture industry due to mortality and reduced growth rates due to a reallocation of resources away from growth. To aid in understanding the energetic costs associated with disease in fish, this study aimed to quantify the energetic costs associated with the immune response in zebrafish (*Danio rerio*). The action of the immune response in zebrafish has been a target of many studies. However, the energetic demands and the behavioural changes involved in the immune response are poorly understood in any fish species. Zebrafish acclimated to 22°C and 27.5°C were either not injected or injected intraperitoneally with 10µl of saline, or heat-killed *Vibrio anguillarum* ($1.21x10^{10}$ cfu/ml). The fish were then placed into respirometry chambers and were acclimated for 3 hours prior to a 24-hour measurement period of their routine metabolic rate (RMR). Following the 24-hour measurement period, the zebrafish were euthanized, and their spleens were collected for RT-qPCR analysis of metabolic and immune related genes. Additionally, in order to examine the energetically costly behavioural responses to an immune challenge, zebrafish acclimated to 22°C were placed into a choice tank, where one side was 22.5°C and the other was 27.5°C and allowed to acclimate for 12 hours and then their movement was recorded for 12 hours. After the 12-hour recording period the zebrafish were injected with 10µl of saline or heat-killed *V. anguillarum* and

placed back into the choice tank for 24 hours (12 hours to acclimate and 12 hours recorded). During the recording periods, temperature preference and the total distance travelled within the choice tank were measured. This study showed that zebrafish at 27.5°C and 22°C injected with heat-killed *V. anguillaum* had an elevated routine metabolic rate compared to fish that were not injected or injected with saline. At 27.5°C there was found to be a 29% increase in RMR and a 20% increase in RMR at 22°C. At 22°C there was found to be a significant increase in *il-1b*, *il-10*, *il-8*, *tnfa*, and *ampka2* transcript abundance in the group injected with heat-killed *V. anguillarum* compared to the other two treatment groups. Analysis of the distance the zebrafish moved after injection with either saline or heat-killed *V. anguillarum* demonstrated that zebrafish movement did not change after the injection of heat-killed *V. anguillarum*. Overall, this study demonstrated that the energetic demands of zebrafish presented with an immune challenge increased. Understanding the metabolic and behavioural changes associated with an immune response will add to our understanding of care for infected fish in the aquaculture industry.

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Chapter 1: General introduction

1.1: Fisheries and Aquaculture

The idea of stocking ponds intentionally with a certain species of fish is not a new or recent idea. It has been used for many centuries around the world and has its roots in China[1]. What is recent, is the wide spread dependence and growth of aquaculture around the globe, especially in China. China now produces more finfish in aquaculture than the rest of the world combined[2]. The trend of increasing production of fish in aquaculture is necessary to support the world's population. The fish stocks in the oceans and inland lakes would not be enough to sustainably meet the demand of the world's fish needs[2]. This trend is shown by the fact that the total weight of fish from capture fisheries has plateaued around 85 million tonnes since 1990 and the total mass of fish produced from aquaculture has been increasing greatly since 1990 and does not appear to be stopping soon. The first year that the total mass of fish from aquaculture was greater than the total mass of fish from capture fisheries was in 2016[2].

In Canada, aquaculture is also incredibly important to meet our food needs and as a means of economic growth and job creation. No longer are we able to solely rely on capture fisheries even when we are bordered by three different oceans and have access to many of the largest lakes in the world. Our need for other methods of fish production is highlighted by the collapse of the northern cod stocks in the 1980s-1990s that destroyed the economy in Newfoundland and Labrador. Over 35,000 jobs were lost due to the moratorium placed on northern cod fishing, this is still the largest layoff in Canadian history[3,4]. Even though the northern cod stocks have increased over the last 20 years, the population is still not stable or close to the point it once was, and the province is now investing in farmed options for northern cod in an attempt to bring the cod market back to Newfoundland[5]. Additionally, the Great lakes

have also suffered from whitefish population declines in the 1960s and 1970s, due to overfishing, introduction of invasive species and habitat destruction[6-8]. These examples and many others have led Canadians and governmental organizations look to more responsible management of natural fish populations and to other sources of fish production such as aquaculture.

In Canada as a whole, the most farmed species of fish is Atlantic salmon. With the majority of production coming from British Columbia[9]. The total mass of Atlantic salmon that was produced from aquaculture in Canada in 2017 was 120,553 tonnes worth just over 1 billion dollars[10]. The total revenue from all shellfish and finfish in Canadian aquaculture is 1.39 billion dollars meaning salmon accounts for ~70% of the revenue in aquaculture in Canada[10]. Ontario though, accounts for the majority of freshwater fish production in Canadian aquaculture. The most commonly farmed fish in Ontario is rainbow trout. In 2017, Ontario produced 5500 tonnes of trout with a production value of $31 million. In total, Ontario produced $34 million worth of fin and shellfish in 2017[9-11]. In both Ontario and Canada, aquaculture production is on the rise. Ontario went from producing 3790 tonnes of finfish and shellfish worth $19.2 million in 2013 to 5900 tonnes worth $34 million in 2017, and represents a 77% increase in production value in 4 years in Ontario. Canada wide, the production increased from 167,000 tonnes worth $870 million to 191,000 tonnes worth $1.4 billion. This was a 60% increase in production value even though the weight only increased by ~15%, largely due to an increase in salmon prices[10,12].

Even though aquaculture can be a profitable business with a clear demand across the world there are still many challenges and drawbacks. Some of these challenges include creating the infrastructure, preventing fish from escaping and reproducing with wild populations, and disease outbreaks. Disease outbreaks are relatively common in aquaculture and this is due to a number of factors. First, the density of fish in aquaculture farms is much higher than what occurs

naturally in oceans and lakes. This causes an increase in interactions between fish, so diseases can spread much more easily and it also favours the spread of more virulent strains of bacteria[13]. Second, the fish in the aquaculture farms are not usually from the native stock. The fish may either not normally found in the lake or environment, or if the specific species of fish being farmed is found in the environment the ones in the aquaculture farm are likely not from the native stock[14,15]. This means that the fish did not co-evolve with the bacteria and viruses in the environment in which they are placed. This makes the fish much more susceptible to infection by the pathogens in the water increasing the risk of infection[16,17]. Lastly, decreased water quality can be an issue because of the high stocking density and single species of organism in very close proximity. It is common for systems to have a recirculating water supply; if the filtration system malfunctions or is not properly cared for, the water could be become high in ammonia, low in oxygen, or become more turbid[18]. A decrease in the water quality can lead to the fish being more vulnerable to disease and aiding opportunistic bacteria in infecting the fish population. Some bacteria appear to do really well in these types of situations like the gram-negative bacteria *Vibrio anguillarum*[19].

Overall, disease has a huge economic impact on aquaculture. The majority of the cost does not come from disease itself but rather from disease prevention[20]. Choosing to focus on Atlantic salmon, since it is the majority of Canada's production in the aquaculture industry; there was an outbreak of infectious hematopoietic necrosis virus (IHNV) in 2001-2003 in British Columbia. During this time about 12 million salmon died either from the infection or needing to be culled to prevent further spreading of the virus. This worked out to be about half of the salmon production in the infected farms during this time period[21]. Clearly epidemics like the one in 2001-2003 are very costly when so many fish die, but preventative maintenance has a high

reoccurring cost. After the outbreak the larger farms installed disinfection systems that cost up to 4 million USD. Additionally, the farms in British Columbia are also screening for IHNV and vaccinating against it with an estimated annual cost of $4.2 million USD[20]. This is just one virus that can cost the aquaculture industry. Another challenge for salmon is sea lice. It is estimated that in Canada sea lice prevention and reduced growth rates from sea lice, cost the industry an extra 15 cents per kilogram of fish for salmon[22,23].

It is likely that disease will start to be handled more efficiently in aquaculture in future years. The aquaculture industry is advancing with new techniques that boost the immune system of the fish in aquaculture for example; probiotics[24]. Hopefully better preventative measures will increase the overall yield and reduce the operating costs for aquaculture farms. Advancements in preventative measures are coming off the back of increased knowledge of the fish immune system. However, an area that is important and has had very little attention brought to it is the balance of energetics and the immune response. A better understanding of the energy needs of the immune system as well as the energy balance and behavioural changes that come with the immune system could help in management of infected fish in terms of optimizing energy to limit the amount of growth reduction from the given injection.

1.2: Immune response in fish

1.2.1: General overview

Zebrafish have become a model organism to study many diseases and have become an effective model for the study of immune responses[25]. For this reason, the zebrafish immune system has been extensively studied. Overall the teleost immune system is very comparable to the mammalian one[25]. Zebrafish have both an innate and adaptive immune response that can

recognize numerous pathogens and foreign molecules[25]. One difference between the mammalian immune system and the teleost immune system is the additional isoforms for some of the proteins. Teleosts have undergone an additional round of whole genome duplication which has led to additional paralogs of a number of genes[26]. Additionally, teleosts are in a different environment compared to most other vertebrates and mammals. Most fish live exclusively in the water and this environment puts them in much closer and frequent contact with many bacteria and viruses[27].

1.2.2: Innate immunity

The innate immune system's first line of defence against infection is the integument. This is a physical barrier that separates the outside environment from the internal environment of the fish. It also keeps microbes from the entering the organism and causing infection[28]. This first line is very important because if the microbial communities cannot interact with the internal environment of the fish, it makes it extremely difficult to infect the host. The integument also has a layer of mucus covering it over the body. This mucosal layer is constantly being shed and replaced on the fish[27]. The mucosal layer not only aids in preventing bacteria from binding to the skin of the fish, but it also contains many antimicrobial proteins and agents that can kill or damage foreign pathogens when they try infecting the fish. The defence mechanisms in the mucus include: antimicrobial peptides, proteases, lectins, and lysozymes[27]. These proteins and enzymes can either kill the foreign microbe (antimicrobial peptides, proteases, and lysozymes) or they will prevent adherence (lectins) of bacteria to the skin of the fish. This aids in keeping foreign bodies outside of the fish[27].

If foreign antigens can get through the integument in fish, there are still innate defences internally in the organism as well. First, the external pathogen needs to be recognized as harmful. Cellular receptors, such as toll-like receptors (TLRs) in the cell membranes of immune related cells can recognize conserved domains and structures of pathogens. These are called pathogen associated molecular patterns (PAMPs)[29]. PAMPs are key parts of the pathogen that the pathogen cannot readily mutate or change to avoid detection by the cell receptors. Some PAMPs for bacteria include lipopolysaccharides, lipopeptides, and flagellin[30]. Two major cells of the innate immune response are neutrophils and macrophages[31]. Neutrophils and macrophages are phagocytic cells, they are capable of engulfing and destroying foreign pathogens typically with reactive oxygen species from respiratory burst[31]. The complement system is one of the major defence mechanisms that are constitutively expressed within the serum and plays an important role in recognition of pathogens and the subsequent opsonization of the non-self-molecules to be more readily phagocytized by neutrophils and macrophages[32]. Another component of the innate immune response are cytokines. Cytokines are small proteins that are released by activated macrophages to communicate with the other cells and macrophages of the body[33]. Some examples of cytokines released by macrophages include; interleukin 8 (Il-8), interleukin 1 beta (Il-1b), tumor necrosis factor alpha (Tnfa), and interleukin 10 (Il-10). Il-8 is a chemokine that participates in chemotaxis recruiting more macrophages to a site of infection[33]. Tnfa and Il-1b are pro-inflammatory cytokines that play a role in the activation of the inflammatory response. In inflammatory response, the area where macrophages converge under direction from chemokines swells due to the inflammatory response and becomes more permeable allowing for the transition of immune cells from the blood to the tissues[33]. Inflammation is important but also needs to be controlled as to not get out of hand[33]. Control of inflammation can be done with the release of

anti-inflammatory cytokines such as interleukin 10 (Il-10). Anti-inflammatory cytokines allow for the limitation and resolution of inflammation after the danger has passed[33]. Macrophages are very important for the innate immune response in signaling and removal of foreign antigens, but they are also very important to the adaptive response as they can be antigen-presenting cells to present antigens to cells of the adaptive immune response[34].

1.2.3: Adaptive immunity

Fish, like mammals have an active adaptive immunity. The adaptive immune response is much more specific compared to the innate immune response[34]. The adaptive immune response also takes more time to be able to respond to a specific pathogen, but it also has a memory so that it can respond quicker if there is a second exposure[34]. For, this reason the adaptive immune response was not a focus of this study because this study focused on a short time frame (27 hours) so, the innate immune response would be more relevant in this time frame.

1.2.4: *Vibrio anguillarum* infection

Vibrio anguilllarum is a gram-negative bacterium that has been found to infect many fish species around the world ex; rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), and Atlantic cod (*Gadus morhua*)[35-37]. It is an opportunistic bacterium that takes advantage of fish in poor health and living in poor environmental conditions[19]. *V. anguillarum* can live in a wide range of temperatures from 0-37°C[38]. The temperature that it is most virulent at was found to be 15-18°C for cold water strains and 20-28°C for warm water strains[38]. *V. anguillarum* infection can cause vibriosis in its host which can lead to death of the fish[39].

Since, *V. anguillarum* is a gram-negative bacterium it has PAMPs that can be recognized by cell receptors within the host. For example, Toll-like receptor 5 (TLR5) in zebrafish has can recognize flagellin of *V. anguillarum*[40,41]. When TLR 5 has recognized flagellin from *V. anguillarum* a signaling cascade will be induced through the myeloid differentiation primary response 88 (MyD88) dependent pathway[42]. The end of the MyD88-dependant signaling cascade is the activation of the protein complex nuclear factor kappa-light-chain-enhancer of activated B cells (Nf-kb)[42]. Nf-kb is responsible for activating transcription of cytokines, such as TNFa, following translocation into the nucleus and binding to NF-kB promoter [42].

Another way that *V. anguillarum* can be detected in zebrafish and a subsequent activation of the immune response is through the alternative complement system[34]. In this response production of the C3b factor of the complement system in the presence of bacteria can stimulate the alternative complement pathway[43]. C3b and the alternative complement system aid in providing opsonization of the bacteria leading to increased phagocytosis of the bacteria[43].

1.3: Energetics in fish

1.3.1: Fish metabolism and respirometry

Proper energy balance is important for all organisms to allocate resources to where they are needed, as well as to keep a reserve of available energy to meet unexpected energy demands that are not routine. Routine energetic demands are described as basic biological functions that are necessary for life and day to day activities[44,45]. Some examples of routine energetic demands for fish are; synthesizing sexual hormones and egg development, spontaneous locomotion, and growth. These routine tasks are what raise the metabolic rate from the standard metabolic rate[46]. The standard metabolic rate is the absolute minimal amount of energy that the fish requires to be

alive[44,45]. It is difficult to measure a fish's standard metabolic rate as it would have to be completely stationary and in a state of sleep. For this reason, the routine metabolic rate (RMR) that includes the minor additional energetic demands of being alive is the most frequently used measurement for energetics in fish[46]. The energy demands that are not routine are often intermittent and high intensity. Examples would include rapid swimming to escape a predator, habitat disruption, or intense migrations. These external stimuli are capable of pushing fish to their limits or their maximal metabolic rates (MMR)[47]. Because most fish are ectothermic poikilotherms, their body temperature will fluctuate with the water temperature. This will affect both their RMR and their MMR depending on whether the fish is close to or far away from its thermal optimum temperature[45,46]. The difference between the RMR and the MMR is known as the aerobic scope (AS) (Fig 1.1). This difference is viewed as the amount of available energy to respond to external stimuli and stressors for survival[45,48]. The AS will vary depending on the temperature of the water with the highest AS being at the thermal optimum for the given fish species. As the temperature moves away from the thermal optimum the AS will decrease[48]. A decrease in AS can come from an increase in RMR without an increase in MMR, through a decrease in MMR with no decrease in the RMR, or it can come from an increase in RMR with a decrease in MMR pinching from both sides (Fig 1.1). Factors that can reduce the AS of fish include; metals in the water, personal care products, hypoxia, and temperature[49-52]. A heathier fish or a fish in cleaner water will often have a large AS allowing for more energy to respond to stressors and environmental stimuli.

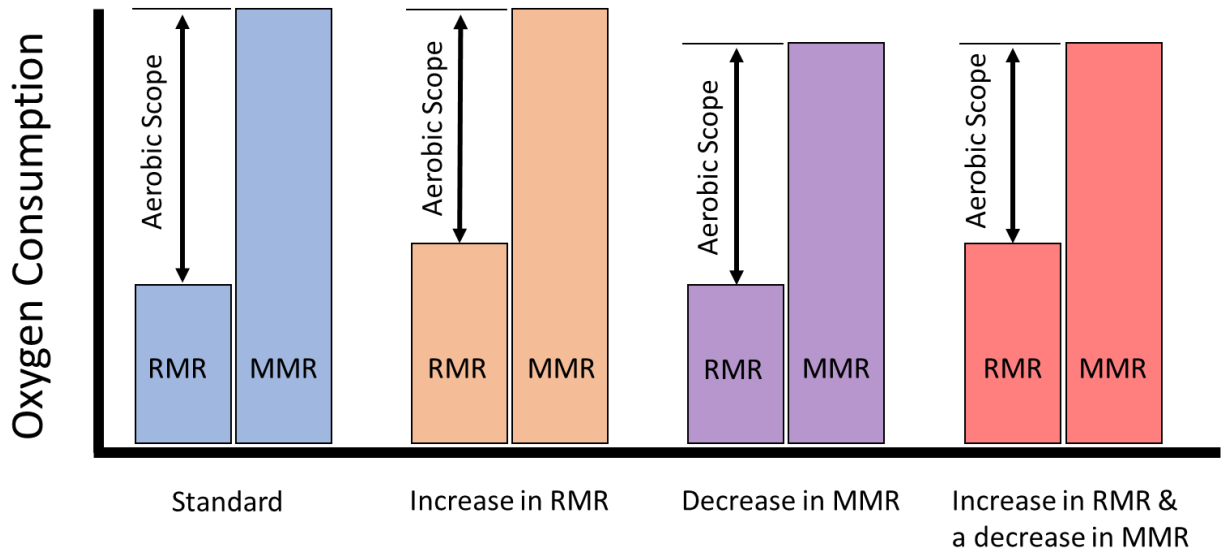


Figure 1.1: **Visualization of aerobic scope** Changes in the routine metabolic rate (RMR) and maximum metabolic rate (RMR) that would decrease the aerobic scope.

To measure the RMR or MMR of fish, respirometry is used. The basic idea behind respirometry is to measure the decrease in oxygen content of the water in a small chamber that a fish is placed in. The decrease in oxygen content is attributed to aerobic respiration of the fish. The amount of oxygen consumed allows for estimation of the metabolism of the fish since oxygen is the final electron acceptor in the electron transport chain (ETC)[53]. Thus, if there is an increase in oxygen consumption rate, then there is an increase in the fish's metabolism.

There are three different techniques to measure the decrease in oxygen content in the water for respirometry. The first, is open chamber respirometry. In this technique oxygenated water is continually flushed through the respirometry chamber and the oxygen content of the water is measured just before the water enters the respirometry chamber and a second sensor is used to measure the oxygen content of the water immediately as it leaves the chamber[54]. In this technique you need to know the flow rate of the water to calculate the amount of oxygen that is consumed over time. This technique is useful because it keeps a continual supply of new

oxygenated water in the chamber, so you do not need to worry about hypoxia or a buildup of waste from the fish. The downside though is that the measurements are less accurate because you are using two different oxygen sensors that each have inherent error[54]. The next technique is closed chamber respirometry. In this technique, only one oxygen sensor is used that continually measures the oxygen content of the water. In this technique the water can be circulated in a closed loop or not circulated at all. This technique gives accurate measurements as it is easy to record the decrease in oxygen content over time and the error of the sensor is not compounded by having 2 different sensors. However, because new water is not being brought in to the system, hypoxia and a buildup of waste can become an issue that could change the respiration rates of fish[54]. The last technique combines the other two techniques to eliminate the drawbacks of the previous two. This technique is called intermittent closed chamber respirometry. Here the respirometry goes through a cycle with three different periods. The first part of the cycle is the flush period. In this phase fresh water is pumped into the respirometry chamber to re-oxygenate the water and remove the waste that was excreted by the fish. The second phase is the wait period. Here, the flush pump is turned off stopping fresh water from being pumped in to the respirometry chamber making it a closed system. The wait period allows the oxygen sensor time give accurate readings to the sudden decrease in the flow of the water[54]. The last phase is the measurement period. This is the only phase in the cycle where the oxygen content of the water is monitored and is typically the longest phase of the cycle. The oxygen content is measured for a given duration overtime. At the end the cycle repeats with the flush period. Intermittent closed chamber respirometry solves the problem of reduced accuracy in open chamber respirometry. It also solves the issue of not being able to keep a fish in the chamber for an extended amount of time in closed chamber respirometry due to the creation of a hypoxic environment. There are

some draw backs as well though with intermittent closed chamber respirometry though. Intermittent closed chamber respirometry generally has a shorter measurement period so, if the fish has a spontaneous increase in activity during the measurement period it may be difficult to get a good R^2 value and a higher RMR will be recorded. For this reason many cycles are needed to get an accurate reading of the RMR for a fish.

1.3.2: AMP-activated protein kinase (AMPK)

Energy balance is a key factor to consider for organisms. Since resources can be limited in the environment, energy needs to be conserved, strictly regulated, and able to respond to different stimuli. For example, if there is a sudden need for a large quantity of energy for movement, there needs to be an appropriate amount of energy reserves so that the organism is capable of responding appropriately. If the increase in energy demands is chronic, non-essential processes need to be inactivated to conserve the available energy. One protein that is capable of doing this is the master regulator of metabolism, AMP-activated protein kinase (AMPK). AMPK is a tri-meric energy sensing protein [55,56]. The three subunits are the α, β, and γ subunits. The α-subunit is the catalytic subunit [57]. In order for AMPK to be activated though, it needs to be phosphorylated by liver kinase B1 (LKB1) at threonine 172 (Thr-172) in mammals [58,59]. Thr-172 is highly conserved in all vertebrates where Thr-172 either exists or there is an analogous threonine that is phosphorylated. Teleost fish have Thr-172, that, when phosphorylated activates AMPK[60]. Once AMPK is phosphorylated at Thr-172 it can start to phosphorylate other proteins such as glucose transporter 1 (GLUT1), glucose transporter type 4 (GLUT4), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), and acetyl-CoA carboxylase (ACC1)[61-65]. The β subunit serves a more structural role. It provides the scaffolding to connect

the α and the γ subunits together and aids in conformational changes that occur when Thr-172 is phosphorylated and when the cystathionin-β-synthase (CBS) domains on the γ subunit are bound to AMP[66]. Additionally, the β subunit also has a carbohydrate binding module (CBM). This domain is responsible for AMPK's interactions with glycogen and potentially the localization of AMPK[67,68]. The γ subunit is also very important as it contains four potential binding sites for adenylates. These four sites are known as cystathionin-β-synthase (CBS) motifs. The four CBS motifs pair together to form two Bateman domains. AMP and ATP theoretically should be able to bind to all four CBS motifs, but one appears to be inactive and the another one of the four is always bound to AMP[66]. This means that the response of AMPK to the [AMP] is based on only two of the four CBS motifs. The AMPK pathway monitors the relative concentration of AMP: ATP [69]. When the [AMP] increases, AMP now out competes ATP at binding to the CBS motifs of the γ-subunit. Binding of AMP to the Bateman domains causes a conformational change in AMPK. This conformational change leads to a greater affinity of Thr-172 to phosphorylation[70]. The combination of AMP binding to the γ subunit and the phosphorylation of Thr-172 leads to greater than a 1000-fold increase in the kinase activity of AMPK[71].

Overall, the main function of AMPK is to monitor the energetic state of the cell by comparing the relative concentration of AMP:ATP. When the [AMP] increases too much in relation to the [ATP], AMPK will become active and begin to activate catabolic processes and inactivate anabolic processes to regenerate the ATP stores of the cell[72]. AMPK does this by being able to phosphorylate a number of different enzymes. Some of the enzymes it phosphorylates to activate catabolic processes include: Acetyl-CoA carboxylase-2 (ACC2), which leads to an increase in fatty acid oxidation[73]; GLUT-1/GLUT-4, which leads to an increase in glucose uptake[61-63]; 6-phosphofructo-2-kinase, which leads to an increase in

glycolysis[74]; and PGC1-α, which when phosphorylated becomes upregulated and stimulates mitochondrial biogenesis[64]. Some enzymes that AMPK phosphorylates to reduce anabolic processes include Acetyl-CoA carboxylase-1 (ACC1), which leads to a decrease in fatty acid synthesis[75], glycogen synthase, which leads to a decrease in glycogen synthesis, and tuberous sclerosis-2 (TSC2), which leads to a decrease in cell growth and protein synthesis[76].

1.3.3 Energetics and the immune response

The immune response and energy balance are intimately connected like most biological processes, but at quick glance it could appear like the two systems are fighting against each other. The immune response needs a quick response to foreign pathogen associated molecular patterns (PAMPs), which involves the synthesis and production of numerous proteins and hormones. The synthesis of immune molecules can lead to a decrease in ATP levels of the cell activating AMPK. The activation of AMPK could lead to inactivation of pathways involved in lipid and protein synthesis[77]. Shutting down protein and lipid synthesis pathways would hinder the immune response if it was unable to produce cytokines or other immune genes[78]. If the organism increased its routine metabolic rate, this would increase the amount of available ATP. With more available energy in the cell, the additional energy requirements for the production of immune related genes would not have as large of an impact on the energetics of the fish. As mentioned above though, an increase in the RMR could reduce the AS if the MMR does not increase as well. In fish however, there is very little known about the energy cost of the immune response.

The energy increase or lack thereof due to the immune response has not been well characterized in fish. There have been several studies performed on mammals looking at the

energy demands of the immune response. Studies in rodents, such as c57BL/6J mice (*Mus musculus*), brandt voles (*Lasiopodomys brandtii*), and tuco-tucos (*Ctenomyidae Talarum*), found that there was a significant change in the oxygen consumption rate (mice and tuco-tucos) and the RMR (brandt voles) of the rodents after being immune challenged[79-81]. Additionally, the study on the brandt voles found that the size of the voles' testicles and epididymis decreased in size over the experiment period while mounting the immune response[80]. This demonstrates that not only has the immune response been shown to increase the overall energy demands in mammals, but other physiological processes may suffer as well, such as reproduction. Trade-offs can work both ways though. As shown in male ruffs (*Philomachus pugnax*) a bird species, the immunocompetence for these males was found to be reduced after sub-dermal injection with phytohemagglutinin during the breeding season compared to the winter[82]. This demonstrates that physiological needs are being balanced based on the available energy and the time of year.

The above studies were all on endotherms which, when invoking an immune response, have the added benefit of being able to increase their body temperature to create a fever. The generation of a fever requires additional energy. This means that an organism's metabolism has to increase for the additional energy and heat production[83]. Ectotherms on the other hand do not have the ability to increase their temperature internally. They have to exhibit a fever through behavioural means, (discussed in Section 1.4.2.). Without an internal increase in metabolism to boost its temperature, it is possible that an immune response in ectotherms will not have the same energetic demands as an immune response in endotherms. This was shown in a study on side blotched lizards (*Uta stansburiana*) where there was no change in oxygen consumption rate after the lizards were injected with lipopolysaccharide[84]. Even though the oxygen consumption rates did not increase, it does not mean there was not additional energy demands of the immune

response. There could still be metabolic trade-offs occurring to keep the oxygen consumption rate the same, especially if resources are limited. There could be a decrease in reproductive genes and tissue growth[85-87]. For fish, the picture is not well known on how the whole organism energetics will respond to an immune challenge.

Energy balance and consumption when confronted with an immune challenge has been poorly studied in fish species. It has been shown that the growth rates of fish that are combating an immune response are lower than the growth rates of healthy fish[22,88]. This demonstrates the impact the immune response can have on the energy allocation of the fish, but the total energetic cost of the immune response has not been identified. The fish literature has only implied that there is an energetic cost to the immune response but has not shown definitively what that cost is.

1.4: Behaviour and Immunity

1.4.1: Increased Lethargy

There has been well characterised sickness behaviour in animals. This behaviour includes; lethargy, anorexia and decreased socialization[89]. These changes that come about in the behaviour of animals when they are sick have been found to be associated with the immune response. Pro-inflammatory cytokines have been found to reduce the appetite of animals as well as reduce the amount that they move[89]. Interleukin-I (Il-1) has been found to reduce the appetite of animals when they are undergoing an immune response[90,91]. The behavioural change of reducing movement when presented with an immune challenge makes sense intuitively from an energetics standpoint. Since, the immune response has been shown to require additional energy[79,81], reducing the amount that the animal moves will conserve energy for the immune and febrile responses. The loss of appetite and anorexia that accompanies an immune response makes

less sense from an energetics perspective though. If the animal needs more energy, it would make sense to eat more, not less. There are two reasons to explain the temporary anorexia with the immune response. First, if the animal temporarily does not feel hungry it does not need to go searching for food, which allows the animal to stay in one place and conserve more energy through not moving[89]. Second, animals will sequester iron from the blood plasma and redistribute it into other parts of the body during a febrile response, and has been shown to limit the ability of bacteria to reproduce as iron is important for bacterial growth[92,93]. If an animal were to eat during an infection, this could supply the blood with new iron to be used by the animal and the infection. So, not eating during an immune response aids in both limiting energy expenditure of searching for food and aids in limiting energy resources available to the infection as well.

Research about the febrile response to pathogens has extensively been done in mammals/endotherms[89,94]. Some research has been shown that ectotherms exhibit a fever[95-98]. Few studies have researched the movement behaviours of ectotherms when presented with a febrile response outside of seeking out warmer thermal conditions. It is possible that ectotherms do not reduce their movement when presented with an immune challenge, as movement maybe more important for ectotherms compared to endotherms. Movement may be more important for ectotherms during a fever because they must increase their body temperature through behavioural means. This is known as a behavioural fever and is discussed in the next section. Since they rely on the environment for heat generation, movement is necessary to find the microclimates that are warmer or cooler depending on their needs at the time[96]. Therefore, it may be detrimental for an ectotherm to become more lethargic during a fever as movement is still an important aspect for temperature regulation for a poikilotherm. More research into this area

needs to be done to understand the movement patterns of ectotherms during an immune response.

1.4.2: Behavioural fever

Ectothermic animals are unable to raise their body temperature in response to an immune challenge internally, therefore they must increase their temperature using external means. Ectotherms will instinctively search out warmer microclimates when inducing a febrile response to raise their body temperature, which is known as a behavioural fever. It has been summarized by Bicego et al. that very similar pathways exist between endotherms and ectotherms with the febrile response[99]. The same pathways will trigger a signalling cascade to tell the brain to increase the body temperature of the organisms inducing a fever in ectotherms and endotherms[99]. The difference being that endotherms increase their metabolic rate and create a fever, whereas ectotherms move to a warmer location to increase their body temperature. Behavioural fever was first demonstrated in 1974 by Vaughn et al. in desert iguanas (*Dipsosaurus dorsalis*) after injection with killed *Aeromonas hydrophila*. Since then, behavioural fever has been shown in many ectotherms including; amphibians[100-102], invertebrates[103,104], and reptiles[96,105,106] and fish[97,107].

Behavioural fever was first demonstrated in fish in 1976 on bluegill sunfish by Reynolds et al.[97]. They demonstrated that the bluegill sunfish injected with killed *Aeromonas hydrophila* in saline, preferred water that was 2.7°C warmer than the group that was injected with just saline[97]. Since 1976, behavioural fever has been demonstrated in many other fish species as well, such as goldfish, zebrafish, pumpkin seed sunfish, Trinidadian guppies and rainbow trout [95,98,107-109]. On average the preferred temperature increase is 2-4°C for a behavioural fever in fish. This

behavioural response to an immune challenge has been shown to dramatically improve the survival rate of fish and the rate in which the infection is cleared[95,107,110]. Even though behavioural fever has been shown to be beneficial in boosting immune response it does not necessarily mean that behavioural fever only has benefits. There are some drawbacks associated with the behavioural fever response as well, which include an increase in metabolic rate as temperature also leads to an increase in RMR increasing the energy demands of the organism. A second drawback is that moving to a warmer destination could also mean moving to a more dangerous location, as the warmer microclimate may have more hazards. For example, larger fish moving to shallower water (which is also warmer) increase the risk of being predated on by mammalian predators[111].

1.4.3 Temperature and immunity

Temperature can play an important role in immunity as it affects many variables that can lead to infection. These variables include; the pathogen's ability to infect, the ability of the pathogen to replicate, the ability of the host to induce an immune response, and the behaviour patterns of the host[112-114]. When all of these factors are taken into consideration there will be a temperature where a specific organism is more susceptible to a specific pathogen. This temperature may be the same or different than the temperature that the host is most susceptible to another pathogen.

Pathogens, like their fish hosts, have an optimal virulence temperature. For example, *V. anguillarum* has a broad thermal preference depending on the specific strain of the bacteria. It can be generally summarized that strains from cooler climates have thermal optimums of 15-18°C and strains from warmer climates of thermal optimums of 20-28°C[38]. Temperatures close

to *V. anguillarum's* thermal optimum are the temperatures where *V. anguillarum* would be the most virulent as these are the temperatures at which it is able to grow the best. Even though pathogens have a thermal optimum, so do fish. A thermal optimum for fish is the temperature where a combination of their growth rate, reproduction, immune response, metabolism and food conversion efficiencies are working the most effectively[115]. For zebrafish, their thermal optimum for growth is around 28.5°C[116]. If an organism moves away from the thermal optimum, then they generally become more susceptible to infection. This is due to an overall reduction in the well-being of an organism as it moves away from its thermal optimum[112,113]. Another factor to consider in the ability of an organism to induce an immune response is the aerobic scope. The thermal optimum is usually where an organism has its greatest aerobic scope and decreases as you move away from the thermal optimum[49]. So, if a fish is in an environment that is higher or lower from its thermal optimum, not only are its enzymes not working efficiently, there is also less available energy to induce a potentially energetically costly immune response[114]. A third factor that contributes to a decrease in immune function is a decreased metabolic rate at lower temperatures. It has been shown that temperatures that are lower than the thermal optimum of an organism can cause a delay in the immune response especially in the adaptive immune response[112,114]. This delay could allow a pathogen enough time to become systemic increasing the difficulty in clearing the infection.

The dynamic of both the host and the pathogen having optimum environmental conditions leads to an interesting balance where it is not enough for the pathogen to be in its ideal environment, but the host also has to be in a weakened state allowing it to be infected. However, the impact of a pathogen when the fish is at a temperature away from its thermal optimum that happens to be the thermal optimum of the pathogen can be lethal to the host[117].

This is because the host is in a more vulnerable environment at the same time the pathogen is in its ideal environment. The balance of host pathogen interactions demonstrates the way that temperature can play a major role in infection and immunity of a host a pathogen.

1.5: Hypothesis and Objectives

1.5.1: Project Overview

As explained above there is an intimate connection between the immune response, the behaviour, and the energetics of an organism. Using heat-killed *V. anguillarum* as the immune system stimulant and zebrafish as the model organism, my research aims to demonstrate and quantify the interconnectedness of the immune response with the regulation of the immune system through AMPK. The foundation of the idea behind the experiments is that injection of heat-killed *V. anguillarum* will stimulate an immune response in zebrafish. The activation of the immune response will lead to the synthesis of many new proteins and hormones involved in the innate immune response, such as Il-1b, Il-6, Il-8, and Tnfa. The sudden and rapid synthesis of molecules will increase the relative concentration of AMP:ATP. This in turn will cause energetic stress on the organism. When this energetic stress, that is the depletion of ATP and buildup of AMP, is detected by AMPK, AMPK will become phosphorylated which will activate and upregulate AMPK resulting in a decrease in anabolic pathways and an increase in catabolic pathways. Using respirometry to measure the oxygen consumption rates between fish that were injected with the heat-killed *V. anguillarum* and the sham injection, differences can be measured and attributed to an increase or decrease in energy consumption due to the immune response.

The energetics might not change just at the molecular level but also at the whole organism level in response to the immune stimulus. One way this change may take place is

through seeking out warmer water which in an ectothermic poikilotherm would increase their metabolic rate as it would increase the organism's temperature. Additionally, the zebrafish may also adjust their swimming behaviour. As it has been shown in mammals undergoing an immune challenge, the distance moved on average in a day decreases when comparing healthy individuals to individuals undergoing immune stimulation[118]. The distance moved for the zebrafish can be simultaneously measured at the same time as the water temperature zone that it chooses to stay in. Lastly, this project aims to repeat the experiment not just at the thermal optimum temperature (28.5°C) of zebrafish but also at a temperature that is below its thermal optimum, where zebrafish are more stressed (22°C)[116]. This is done to be able to compare the affects that temperature has on the balance of the immune response and metabolism.

1.5.2 Predictions and objectives

The objectives of this study were to increase our understanding of the energetic needs of the immune response in zebrafish. Since it has been demonstrated that growth is reduced in fish when they are presented with an immune challenge[22,88]. It is logical to assume that the immune response requires energy and is either taking energy away from other physiological processes such as growth, or it is increasing the routine metabolic rate of the organism to provide the additional energy resources necessary for the immune response. This has never been demonstrated in zebrafish. The second objective of the study was to examine and observe if there were any behavioural changes that would also affect the energetics of the fish. The two behavioural changes that were measured in the study were; the amount of extra time that the fish spent in warmer water over the course of the day and the total distance that the fish moved over the course of the day.

1.5.3 Hypothesis

Zebrafish injected with heat-killed *V. anguillarum* will have a measurable increase in their oxygen consumption rate, with a concomitant increase in energetic and immune responsive transcripts, and their behaviour will change, in which they will prefer warmer water and move less compared to the control group.

Chapter 2: Materials and Methods

2.1 Animals

Adult zebrafish of mixed sex (*Danio rerio*; n=72) were purchased from AQUAlity tropical fish wholesale (Mississauga, Ontario) and Petsmart (Waterloo, Ontario). All zebrafish were housed in aerated and filtered, 20L glass aquarium tanks containing dechlorinated city of Waterloo water. The zebrafish were kept at two separate temperatures of 22°C and 27.5°C. After the fish were at the desired temperatures no experiments were carried out on them for 1 month to allow them to fully acclimate to the new temperatures and tanks. The fish were fed once a day to satiation with gemma 300 fish food, (Skretting, Stavanger, Norway), and fish were maintained on a 12h:12h light dark cycle. All experimental procedures followed the Canadian Council of Animal Care guidelines and were approved by the University of Waterloo Animal Care Council (AUPP #30005).

2.2 Preparation of the heat-killed *Vibrio anguillarum*

All chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich (Mississauga, Ontario). The heat-killed *Vibrio anguillarum* was prepared from a glycerol stock of *V. anguillarum* provided by the Brian Dixon lab (University of Waterloo, Ontario). Three ml of tryptic soy broth (TSB) was placed into two sterile 10 ml test tubes. The TSB was made as per the manufacturer's guidelines with the addition of 15 g/L of NaCl to make the resulting media 2 % NaCl. Using a sterile inoculating loop some of the frozen culture of *V. anguillarum* was scraped into one of the two 10ml test tubes containing TSB. The two test tubes were then labelled as the sterile control and the *V. anguillarum* culture. The test tubes were placed into a shaker and shook for 16 hours at room temperature at 200 rpm. After the 16-hour incubation

period the test tubes were removed from the shaker and the sterile control was visually inspected to see if there was bacterial growth to make sure that the TSB was not contaminated with bacterial growth. The *V. anguillarum* culture was also inspected for bacterial growth. 1ml was removed from the tube containing the *V. anguillarum* culture and placed into a sterile 1.5ml microcentrifuge tube. This tube was then centrifuged for 8 min at 10,000 RPM at room temperature. Following the centrifugation, the supernatant was removed and washed with 1ml of sterile phosphate buffered saline (PBS), and centrifuged again. After the second centrifugation step, the supernatant was removed, and the pellet was completely resuspended in 1ml of sterile saline. A boiling cap was placed on the microcentrifuge tube and then the resuspended sample was placed in a beaker of boiling water for 30 min.

While the sample was being boiled, a standard plate count was done using the remaining culture to determine the number of viable bacterial colonies in the culture. To do this a serial dilution was carried out. 4.5ml of TSB +2%NaCl was placed in to 8 tubes labelled 10^{-1} to 10^{-8}. 500 µl of bacterial culture was added to the tube labelled as 10^{-1} and then 500 µl from each tube was added to the next in a serial dilution using a new tip for each part of the serial dilution. Using the 10^{-6}, 10^{-7}, and 10^{-8}, Concentrations of the serial dilution 100 µl was plated on triplicate tryptic soy agar (TSA) plates +2 % NaCl (15g/L) for a total of 9 plates (3 of each concentration). The plates were left upright for 5-10 minutes to allow the bacterial culture to absorb onto the plate and then they were flipped. The plates were left to incubate on the benchtop at room temperature for 24h.

When the 1ml culture was boiled for 30 minutes it was removed from the water and placed in the fridge (4°C) for 5 minutes to cool. After the sample had cooled in the fridge, 100µl of the sample was plated onto a TSA +2 % NaCl plate. The plate was left upright to absorb for 5-

10 minutes and then flipped and left on the benchtop to incubate at room temperature for 24 hours. The remaining 900µl of the heat-killed sample was centrifuged for 8 minutes at 10,000 RPM at room temperature. The supernatant was then removed, and the pellet was resuspended in a 50:50 mixture of saline and Freund's incomplete adjuvant.

After the 24-hour incubation period of the TSA plates, the plate that was incubating the heat-killed sampled was visually inspected to ensure that there was no bacterial growth on the plate indicating that there were no viable bacteria colonies. The plates from the dilution series were inspected and the concentration that appeared to have a colony count between 75-150 on each of its plates was chosen for the CFU calculation. The 10^{-7} dilution was chosen. The equation for the CFU calculation was:

$$\text{CFU} = \#\text{ of colonies} \div (\text{Sample dilution x Volume plated in ml})$$

All the agar plates, the serial dilutions, and the remaining live sample of the *V. anguillarum* were disposed of as hazardous waste.

2.3 Experiment 1: Acclimation period for zebrafish at 22°C

To determine the amount of time required for zebrafish to acclimate to the confines of the respirometry chamber, an acclimation study was performed. In this experiment, eight zebrafish were placed into respirometry chambers and their oxygen consumption rates were measured over a 24-hour period. Afterwards the data was analyzed to see how long it took for the oxygen consumption rates of the zebrafish to plateau and reach routine levels. This was used as the time point for the metabolic rate measurements (Figure 3.1).

2.4 Experiment 2: Metabolic rate measurements following injection

The zebrafish that were kept at 22°C were acclimated by reducing the temperature of the water by 2°C per day from 27.5°C. The zebrafish were held in their tanks at the desired temperatures for a minimum of 8 weeks prior to the measurement of their oxygen consumption rates. For this experiment zebrafish were treated in one of three ways: 1) a non-injected control group; 2) a control group injected with 10µl of a mixture of saline and Freund's incomplete adjuvant; 3) a treatment group injected with 10µl of heat-killed *Vibrio anguillarum* ($1.15x10^{10}$CFU/ml) dissolved in saline and Freund's incomplete adjuvant. There were 8 fish per treatment and this experiment was conducted at 22°C and at 27.5°C for a total of 48 zebrafish.

Prior to injection all the zebrafish were anaesthetized in 150 mg/L of Ethyl 3-aminobenzoate methanesulfonate salt (MS-222) buffered with sodium bicarbonate. All the fish were injected intraperitoneally. The fish were injected at 9am and then placed into individual respirometry chambers. The fish were kept in these chambers for 3 hours before any the recordings began, to allow the fish to calm down after the handling stressor of being placed in the chamber, as indicated by the acclimation study. Following the 3-hour acclimation period the recordings began. The fish's oxygen consumption rate was measured for 24 hours. Following the 24-hour measurement period the fish were immediately euthanized in a lethal concentration of MS-222 (500mg/L) and spleen, liver, muscle, gonad, and kidney tissues were collected and frozen at -80°C for reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis.

An intermittent flow respirometer from Loligo (Vibørg, Denmark) was used to measure the oxygen consumption rates of the zebrafish. The software used to analyze the data was the

Loligo Autoresp. To calibrate the flow through oxygen sensors, the sensors were placed in a 10L tank with water flowing through the sensor. Oxygen was bubbled into the closed tank for 20 minutes and then a measurement was taken. This measurement was used as the 100% oxygen saturation. For the 0% oxygen content the sensors were placed in a 10L tank of water and nitrogen was bubbled into the water for 20 minutes and a measurement was taken. The cycle for the intermittent respirometry consisted of a 240-second flush period where new oxygenated water was brought into the system. This was followed by a 60-second wait period between the time when the flush pump was turned off and the measurement phase began. This was used as approximately 30 seconds after the flush pump was turned off, the oxygen content measurements of the water could be unreliable. The last phase was a 300-second measurement period. This period measured the decrease in oxygen content within the water in the chamber over the 300-second recording and using the slope the mg O_2 / hour could be calculated.

The oxygen consumption rate of the zebrafish was corrected for multiple factors. First, the measurement was corrected by subtracting the bacterial background respiration within the chamber in mg O_2 / hour. The bacterial background was measured immediately before and after the fish was inside the chamber. The cycle for measuring the bacterial respiration of the water was a 240 second flush period, then a 60 second wait period and ending with a 1300 second measurement period. The longer measurement period for the bacterial background was due to the lower oxygen consumption rate so more time was required to get an adequate R^2 value. The second correction factor was the weight of the zebrafish. All the oxygen consumption rates were divided by the weight of the zebrafish to mass correct the oxygen consumption values. Following the mass correction, the final value was then adjusted using the allometric scaling equation for zebrafish [119]:

$$MO_{2cor}=MO_{2meas}(M_{meas}M_{cor}^{-1})^{1-b}.$$

In this equation MO_{2cor} is the corrected mass specific oxygen consumption rate. MO_{2meas} is the measured mass specific oxygen consumption rate. M_{meas} is the measured mass of the zebrafish. M_{cor} is the standard mass (1g) for the zebrafish. Lastly, b is the allometric scaling constant for adult zebrafish.

2.5 Experiment 3: Behavioural changes associated with the immune response

To measure if there were behavioural changes associated with the immune response in zebrafish, a choice tank from Loligo was used. For this experiment there were two treatment groups. The first group was injected intraperitoneally with 10µl of a 50:50 mixture of saline and Freund's incomplete adjuvant. The second treatment group was injected with 10µl of heat-killed *V.anguillarum* suspended in a 50:50 mixture of saline and Freund's incomplete adjuvant. This experiment was conducted on the fish that were acclimated to 22°C only. There were 12 adult zebrafish per treatment for a total of 24 zebrafish for this experiment.

The choice tank used had two large circular holding tanks that were connected in the middle by a passage tunnel. The fish could freely swim between the two sides of the tank. The one side was kept at 22.5°C and the other side was kept at 25°C. Above the tank was a camera that recorded the movement of the fish using the Loligo Shuttlesoft software. This software tracked the amount of time the fish spent on either side of the choice tank, the position of the zebrafish in each of the sides, the distance that the fish had travelled, and the velocity of the zebrafish.

For this experiment, a fish was placed into the choice tank at 7:30PM and then left alone, overnight (12 hours). This was done so that the fish could acclimate to the new environment. At 7:30AM the following morning the camera would be turned on and begin recording. The observation period would last until 7:00PM. At this point the camera would stop recording the fish and it would be removed from the choice tank. The fish would then be anesthetized in 150mg/L MS-222 buffered with sodium bicarbonate and injected with either the saline and Freund's incomplete adjuvant or the heat-killed *V. anguillarum* suspended in saline and Freund's incomplete adjuvant. Following the injection, the fish was placed back into the choice tank at 7:30PM and would be left alone overnight (12 hours) to re-acclimate to the choice tank. At 7:30AM the next morning the fish were recorded again until 7:00PM. At 7:00PM the fish was removed from the choice tank and euthanized in 500mg/L MS-222. Between each fish a half water change was completed as there was no filter in the set up to prevent the buildup of ammonia.

2.6 RT-qPCR analysis

The frozen spleen tissues from experiment 2 were used for RT-qPCR analysis. The tissues were homogenized using the OMNI tissue homogenizer (Kennesaw, Georgia, USA). Following tissue homogenization, RNA was purified and extracted using the miRNeasy kit from Qiagen (Hilden, Germany). The purification of total RNA, including small RNAs, was carried out as per the manufacturer's instructions. RNA quality was inspected using the 280:260μm and 260:230μm wavelength ratios.

After the extraction of total RNA, the RNA was converted into cDNA using the miScript II RT kit from Qiagen (Hilden, Germany). The quantification of the amount of RNA was done

using the SpectraMax 190 from Molecular Devices (San Jose, California, USA). The reverse transcription was carried out using the 20 µl reaction, the HiFlex buffer and 300 ng of input RNA. The procedure was carried out as per the manufacturer's guidelines.

Following cDNA synthesis RT-qPCR was carried out. Biorad SSo advanced SYBR green (Hercules, California, USA) was used as the DNA stain/dye for RT-qPCR analysis. The instrument that was used was the Biorad CFX 96 touch machine using the CFX Maestro software from Biorad (Hercules, California, USA). The protocol for a 10 µl reaction was followed with 1µl of Forward and reverse primers with a final concentration of 500 nM, 2µl of sample were placed into the machine with a final concentration of 3ng of cDNA. 1µl of nuclease-free water was added along with 5µl of SYBR Green per reaction well. Each reaction was carried out in duplicate in Biorad low-profile clear plates with bio-rad optically clear flat caps (Hercules, California, USA). Primers were validated by using a 5-point dilution series starting with a ¼ dilution and increasing the dilution by a factor a factor of 4 each time. Reference genes were selected based on stability calculated using the Biorad CFX Maestro software (Hercules, California, USA). The reference genes used were: *18s* rRNA, *ef1a, 7sk* small nuclear RNA and *b-actin*. The thermal conditions used were: a 30 sec polymerase activation at 95°C, then a 10 sec denaturation phase at 95°C followed by a 30 sec annealing/extension phase at 60°C. The denaturation and annealing/extension phases were cycled for 40 cycles total. After the amplification steps a melt curve was completed between 65-95°C taking a recording every 0.5°C for quality control.

Table 2.1: **Primers used in this study**

Transcript Target	Accession Number	Amplicon Size	Primer Efficiency	Sequence
18s rRNA	NR_145818	130 BP	107.9	F: ATGGCCGTTCTTAGTTGGTG
				R: GAACGCCACTTGTCCCTCTA
ampka1	NM_001110286	147 BP	107.9	F: AGTTATCAGCACACCGACAG
				R: CAGTAATCCACCCCTGAGATG
ampka2	XM_695739	139BP	107.6	F: CTACATCCCCGAATACCTCAAC
				R: GAACAGGTAGCCAGGAAGATC
il-1b	NM_212844	150 BP	100.6	F: TGGACTTCGCAGCACAAAATG
				R: GTTCACTTCACGCTCTTGGATG
il-8	XM_009306855	158 BP	106.3	F: GTCGCTGCATTGAAACAGAA
				R: CTTAACCCATGGAGCAGAGG
il-10	NM_001020785	235 BP	113.9	F: ACGAGATCCTGCATTTCTACTTG
				R: AGCTCCCCCATAGCTTTATAGAC
nf-kb (rela)	NM_001001840	354 BP	107.4	F: AGTATCCAGTCCATCTCGCTGT
				R: GCTTCTTCTCGCTCTCTTCATC
tnfa	NM_212859.2	76 BP	95.4	F: CCATGCAGTGATGCGCTTTT
				R: CGTGCAGATTGAGCGGATTG
ef1a	NM_131263	134 BP	96.2	F: CAAGGAAGTCAGCGCATACA
				R: TCTTCCATCCCTTGAACCAG
7sk	AJ890102	127 BP	101.5	F: ACGAGCATCGCTGGTATAGAA
				R: GCCTCATTTGGATGTGTCTGA
b-actin	NM_131031	117 BP	97.8	F: CCCAGACATCAGGGAGTGAT
				R: CACAATACCGTGCTCAATGG

2.7 Statistical analysis

All the statistics were completed using Prism 8 from Graphpad (San Diego, California, USA). All data presented is given as the mean ± the standard error of the mean (SEM). To determine the break point for the acclimation, study a segmented linear regression with a gentle

connection was used. For comparison of the oxygen consumption rates over time across the different treatments, a 2-way ANOVA was used. Tukey's post hoc test was used for multiple comparisons of the treatments when differences were found. For the behavioural study comparing the amount of time spent in each of the zones as well as the differences between the distance travelled before and after injection, Shapiro-Wilk's test was used to test for normality. Then either an unpaired t-test was used for normally distributed data or the Mann-Whitney test was used for non-parametric data. To compare the differences for analysis of RT-qPCR, normality was again checked using the Shapiro-Wilk test. After checking for normality either a one-way ANOVA was used to compare the ΔΔCQ values of the RT-qPCR results, or the Kruskal-Wallis test for non-parametric data. For multiple comparisons Tukey's post hoc test was used. For finding significant differences the alpha value was set at 0.05 for all of the statistical tests. So, differences were only considered if the P value was less than 0.05.

Chapter 3: Results

3.1: Acclimation study

A segmented linear regression with a gentle connection was used to determine the break point at which the zebrafish returned to a resting state after the initial handling stressor of being placed into the respirometry chambers. The time point that gave the best R^2 value was used as the break point. It was found that two hours after injection the curve switched from a greatly negative slope (-0.1922mg/O_2/g/hr) to a slope that was essentially zero (-4.639x10^{-5}mg/O_2/g/hr). The R^2 was calculated using the mean oxygen consumption rate at each of the time points.

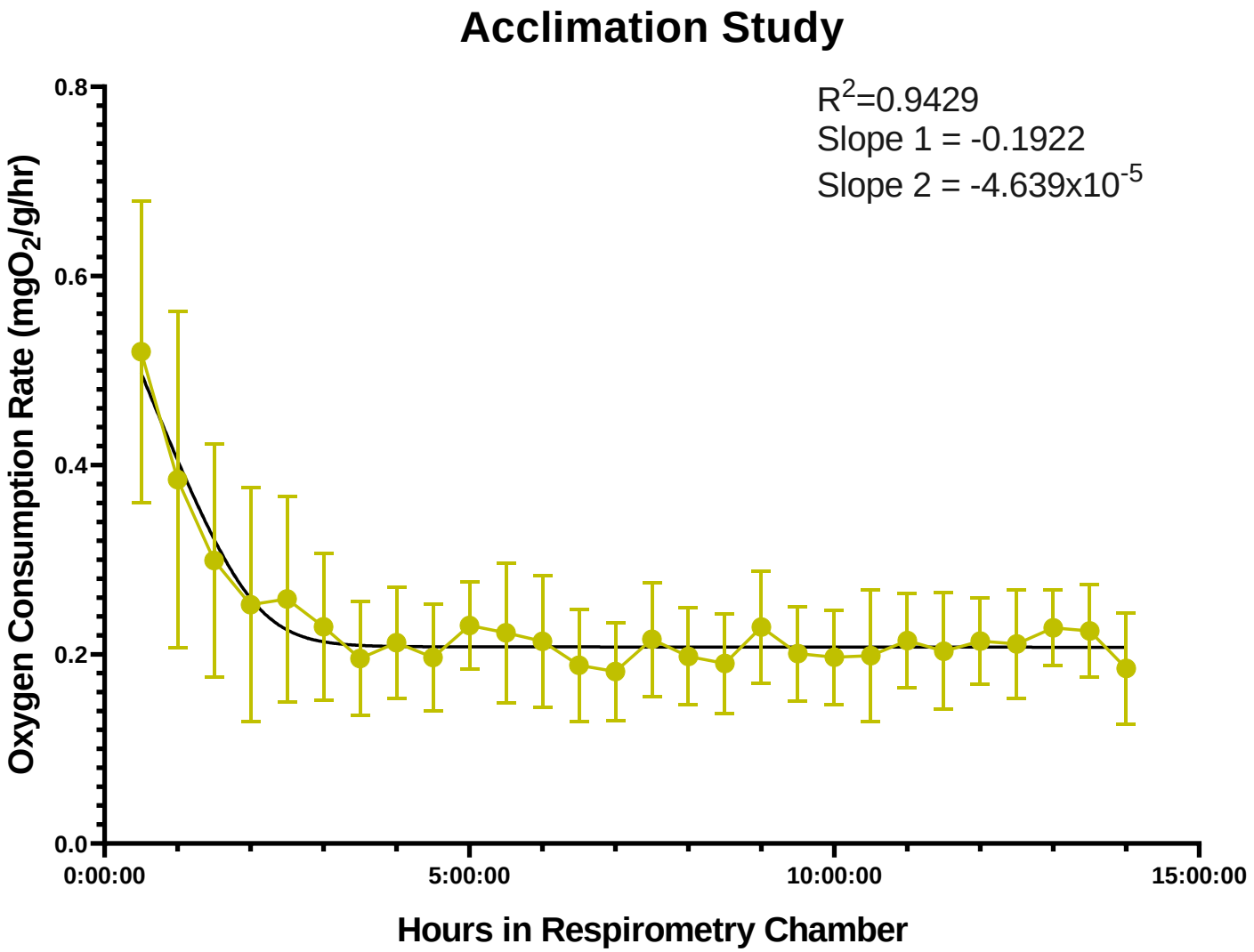


Figure 2.1: **Time required for zebrafish to reach RMR after being placed in respirometry chambers.** Mean oxygen consumption rates ± 95% confidence interval for acclimation study for zebrafish in 30-minute intervals following the handling stressor of being placed into the respirometry chamber. A segmental linear regression with a gentle connection was used to find the break point. The time point for the break point that had the best R2 value was 3 hours after being placed into the respirometry chamber (n=8).

3.2 Oxygen consumption rates

At 22°C the oxygen consumption rates for the non-injected, the saline and Freund's incomplete adjuvant, and the heat-killed *V. anguillarum* groups were not found to be statistically significant using a 2-way ANOVA when looking at the full 24-hour observation period (Fig 2.2). The mean oxygen consumption rate for the non-injected group over the 24-hour measurement period was 0.2692 ± 0.0054 mg O_2/g/hr, for the saline and Freund's incomplete adjuvant group it was 0.2636 ± 0.0041 mgO_2/g/hr, and for the heat-killed *V. anguillarum* group the mean was 0.2940 ± 0.0040 mgO_2/g/hr. The 2-way ANOVA also showed that there were differences over time within all the treatments. Tukey's post hoc test was used to find out where those differences lied. The majority of the differences for all the groups were from comparing the 10.5 and 22.5 hours post injection time points to most of the other time points. These times correlated with when the lights turned off and on again in the aquatics facility (Figure 2.2). A full list of all the differences found between the time points are found in Table A.1 in the Appendix.

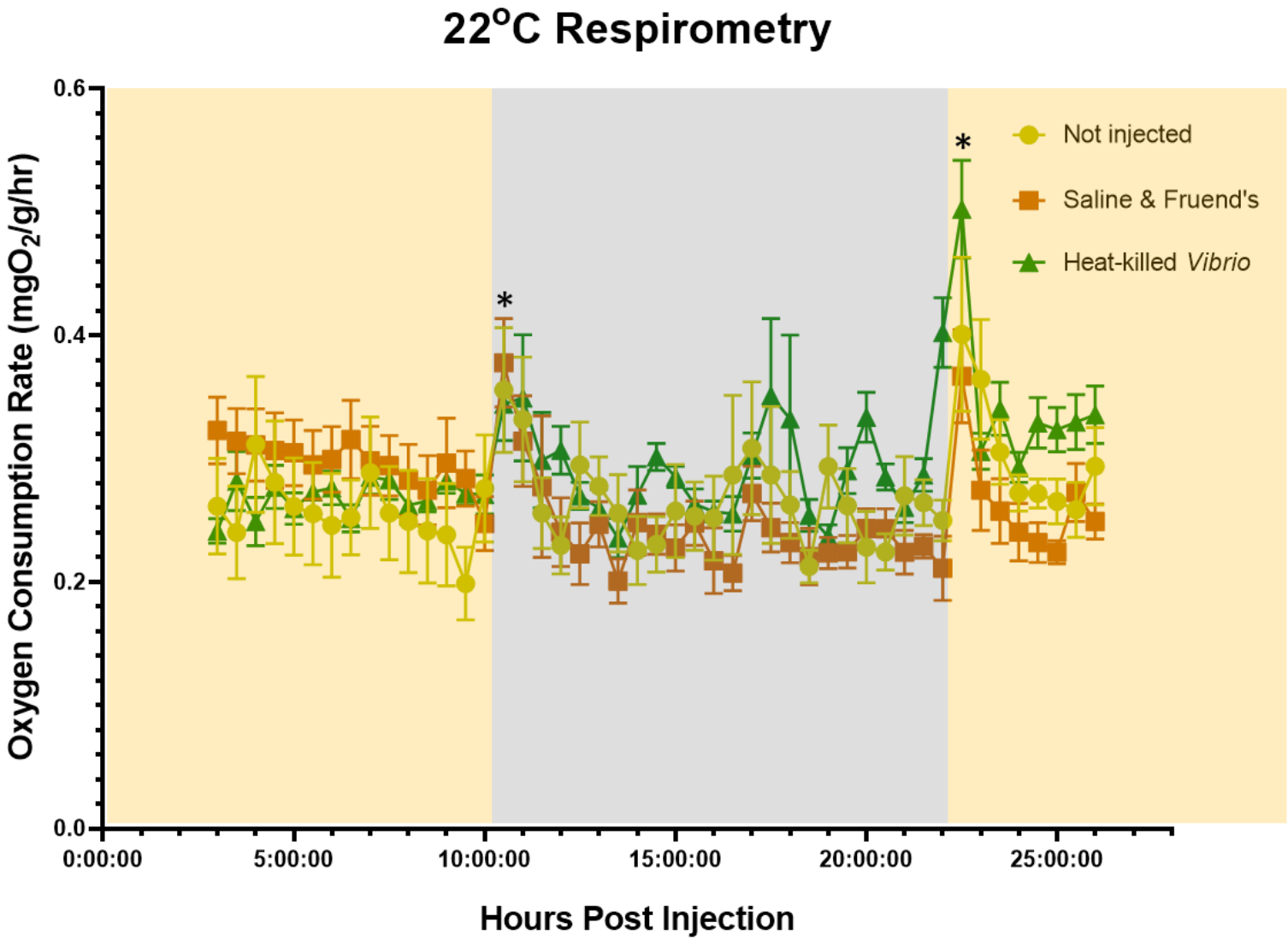


Figure 2.2: Oxygen consumption rates for the three treatment groups 3-27hpi at 22°C. Mean oxygen consumption rates ± SEM over time at 22°C at the different time points after injection for each of the treatment groups. The yellow areas indicate the hours with the lights on and the dark area are the hours with the lights off. A 2-way ANOVA was used to find any difference between time points and treatments (n=8 for each treatment, n=24 for each time point, α=0.05).

However, there appeared to be point at 15 hours post injection where the oxygen consumption rate for the Heat-killed *V. anguillarum* treatment group started to be statistically higher than the oxygen consumption rates for the other two treatment groups (Fig 2.2). All three treatment groups were found to be significantly different from each other when looking at 15 hours post injection onwards. The heat-killed *V. anguillarum* treatment group had the highest oxygen consumption rate with a mean of 0.3110 ± 0.0066mgO_2/g/hr. The group that was not injected with anything had the next highest oxygen consumption rate with a mean of 0.2757 ± 0.0075mgO_2/g/hr and the saline and Freund's incomplete adjuvant treatment group had the lowest oxygen consumption rate with a mean of 0.2427 ± 0.0047mgO_2/g/hr (Figure 2.3). Again, a statistical difference was found between the different time points. Most of the differences were between the 22.5 hpi time point and the rest of the time points. A complete list of the differences between the different time points can be seen in Table A.2. in the Appendix.

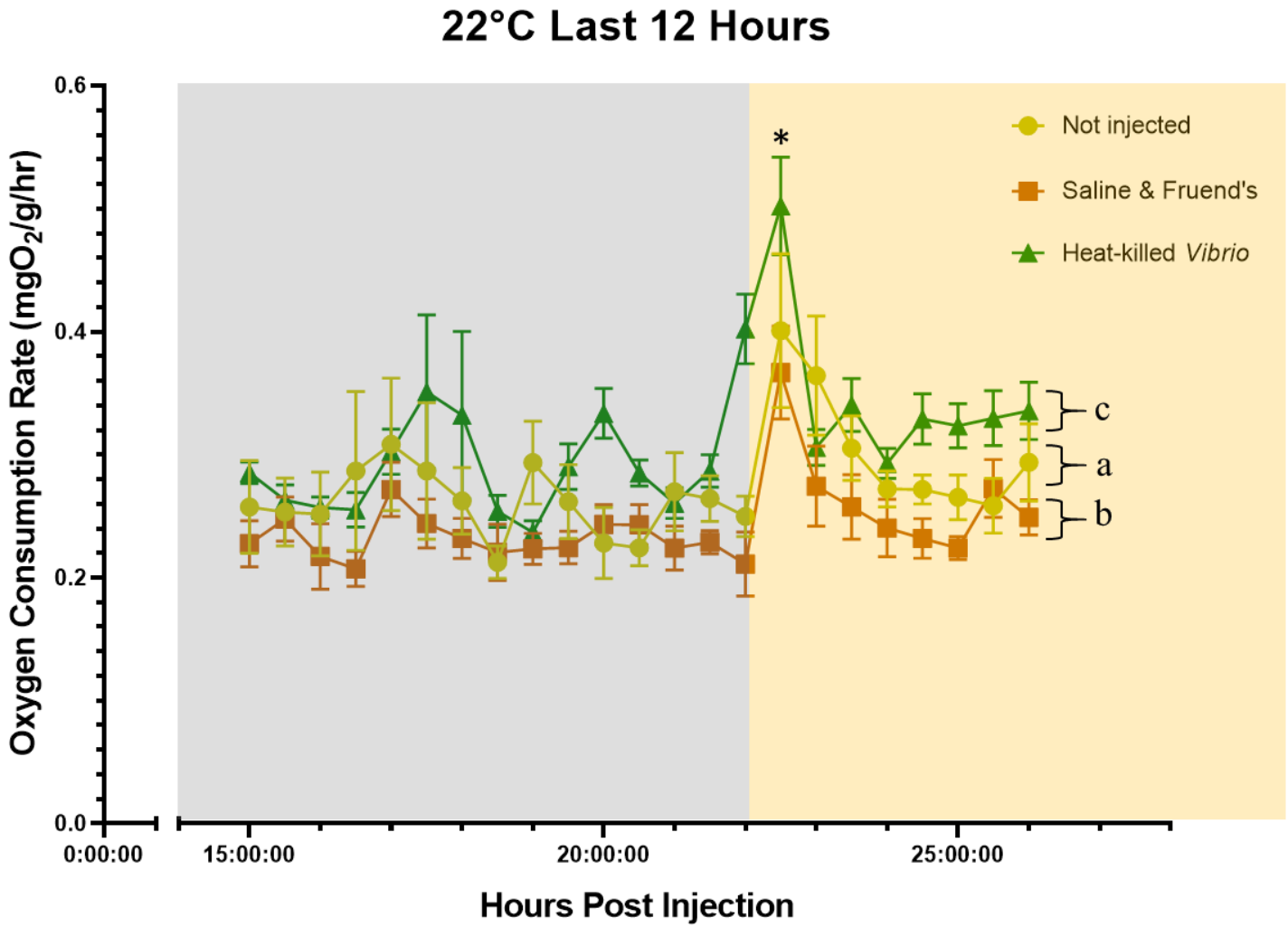


Figure 2.3: Oxygen consumption rates for the three treatment groups 15-27hpi at 22°C. Mean oxygen consumption rates ± SEM over time at 22°C starting at 15 hours post injection for each of the treatment groups. The yellow areas indicate the hours with the lights on and the dark area are the hours with the lights off. A 2-way ANOVA was used to find any difference between time points and treatments (n=8 for each treatment, n=24 for each time point, α=0.05) The oxygen consumption rate for the heat-killed V. anguillarum treatment group was ~20% greater than the other treatment groups.

At 27.5°C the oxygen consumption rates for the non-injected, the saline and Freund's incomplete adjuvant and the heat-killed *V. anguillarum* treatment groups were compared using a 2-way ANOVA. There were significant differences for both the treatment groups as well as differences based on time. Tukey's posthoc test revealed that the heat-killed *V. anguillarum* treatment group (mean = 0.5261 ± 0.0087mgO_2/g/hr) was significantly different than both the non-injected group (mean = 0.4020 ± 0.0051mgO_2/g/hr) and the saline and Freund's incomplete adjuvant group (mean = 0.4123 ± 0.0064mgO_2/g/hr) this works out to be an ~29% increase in oxygen consumption rate for the heat-killed *V. angiullarum* group compared to the other two groups (Fig 2.3). There was no difference between the saline and Freund's incomplete adjuvant group and the non-injected group (Fig 2.4). There were again differences between the different time points. For the 27.5°C group many of the differences were found between the 22 hpi time point and the time points preceding it. All the differences that were found between the time points can be seen in Table A.3 in the Appendix.

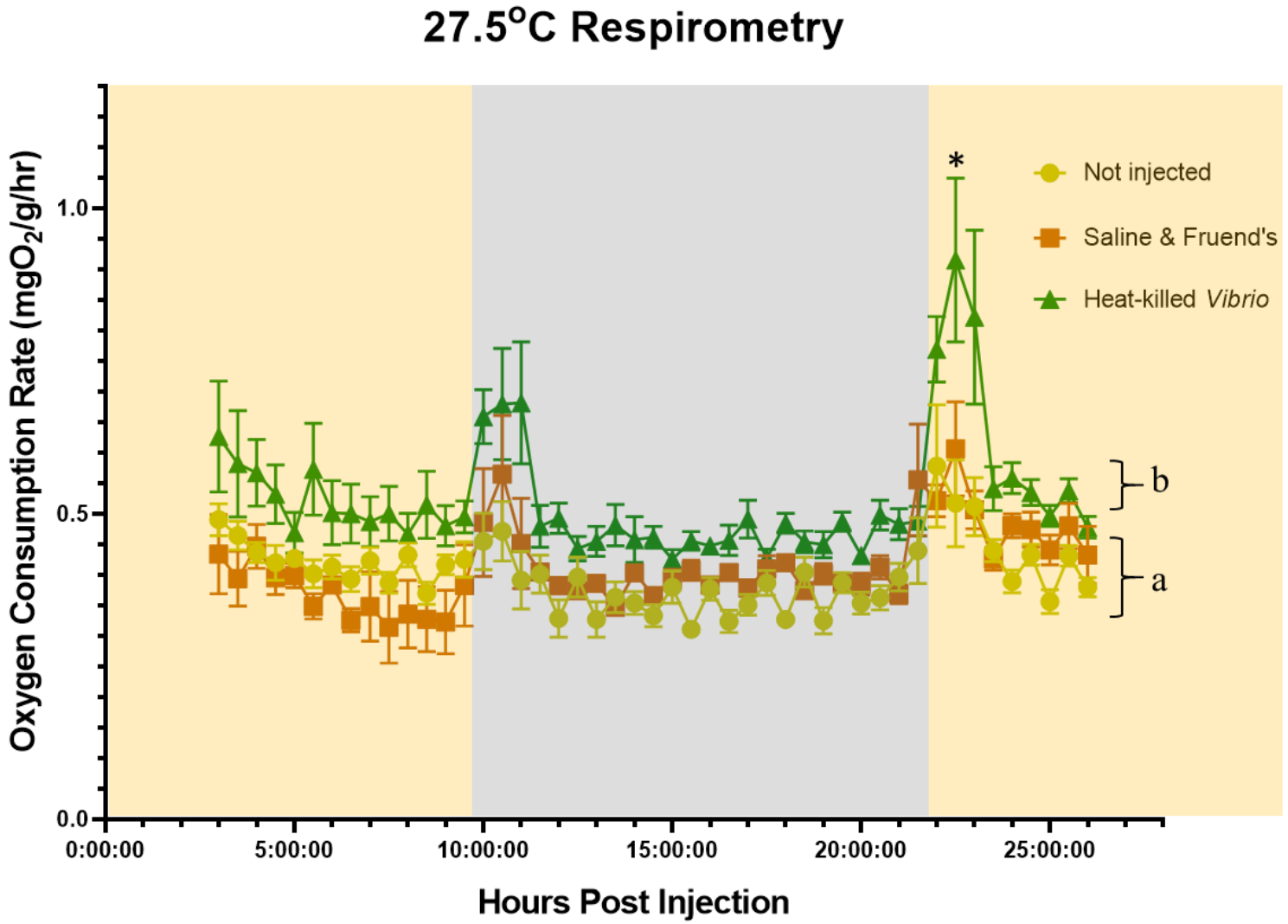


Figure 2.4: Oxygen consumption rates for the three treatment groups 3-27hpi at 27.5°C. Mean oxygen consumption rates ± SEM over time at 27.5°C at the different time points after injection for each of the treatment groups. A 2-way ANOVA was used to find any difference between time points and treatments (n=8 for each treatment, n=24 for each time point, α=0.05). The oxygen consumption rate for the heat-killed V. anguillarum treatment group was ~29% greater than the other treatment groups.

3.3 Behavioural changes

For the behavioural changes associated with the immune response, one of the metrics that was measured was the distance travelled. On average the fish moved about 1464.93 ± 172.72m per day on the first day before injection with saline and Freund's incomplete adjuvant. (Figure 2.5a). After injection with saline and Freund's incomplete adjuvant the fish travelled 1201.96 ± 172.82m (Fig 2.5a). On average before injection with heat-killed *V. anguillarum* the fish travelled 1335.17 ± 185.62m and after injection the fish travelled 1149.23 ± 168.96m (Fig 2.5b). To normalize the data and reduce the impact of individual variability, the distance travelled before and after injection was compared for each fish in both treatment groups (Fig 2.6). There were also no differences found in the relative distance travelled after injection between the two treatment groups after comparison with a t-test (Fig 2.6).

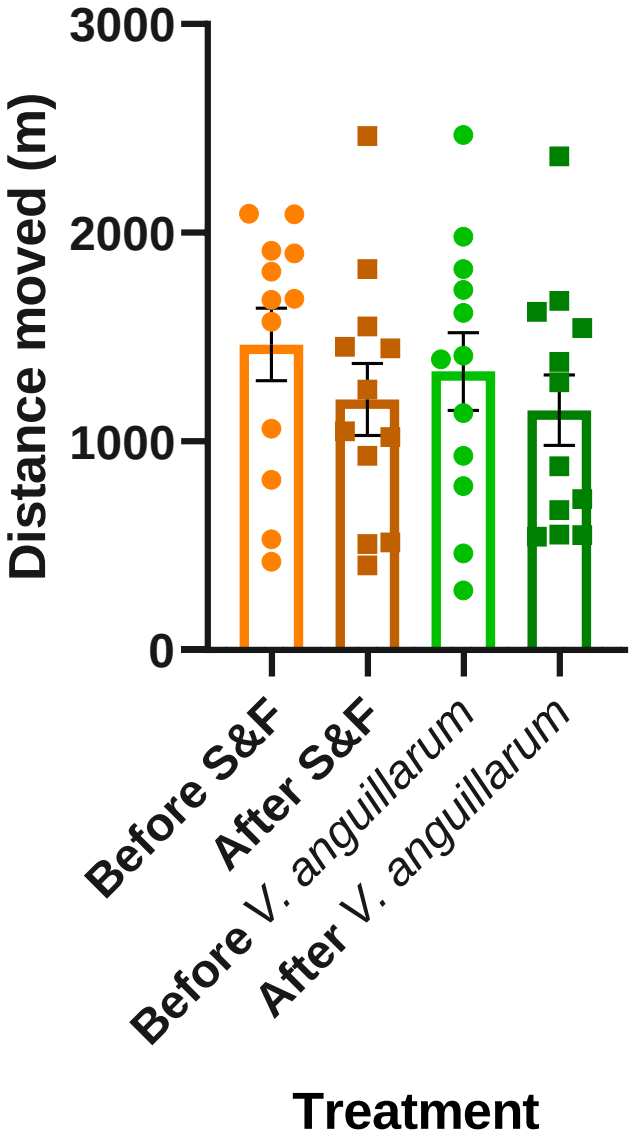


Figure 2.5: Total distance moved (m) over the recording period for both treatments before and after injection. Mean ± SEM values of the distance moved in m for the behavioural study for the various treatments over the course of the 11.5-hour observation period (n=12).

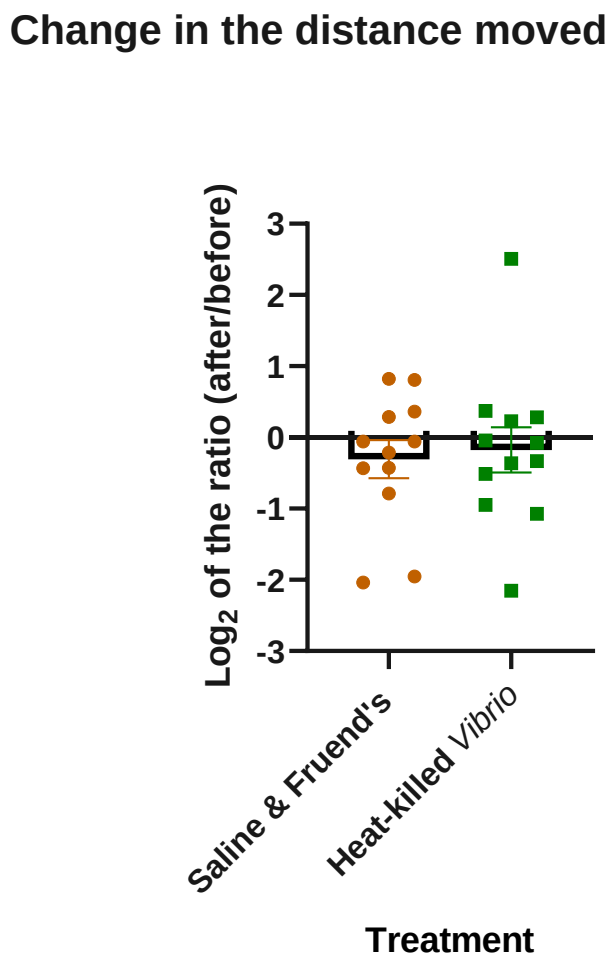


Figure 2.6: Fold change of the distance moved before and after injection for both treatment groups. Mean value of the log fold change of distance moved pre and post injection. Values are presented as the mean ± the SEM (n=12). No statistical significance was found using an unpaired t-test.

Even though the total distance moved was not different between the treatment groups, it was possible that the fish may have moved more on one side of the tank and less on the other. To analyze the change in velocity on each side of the tank, pre- and post-injection, an unpaired T-test for the warm zone and Mann-Whitney test for the cold zone was used. It was found that there was no significant difference in the change in velocity after injection between the two treatment groups (Fig 2.7). For both sides of the tank there was no $\log_2$ fold change in the velocity.

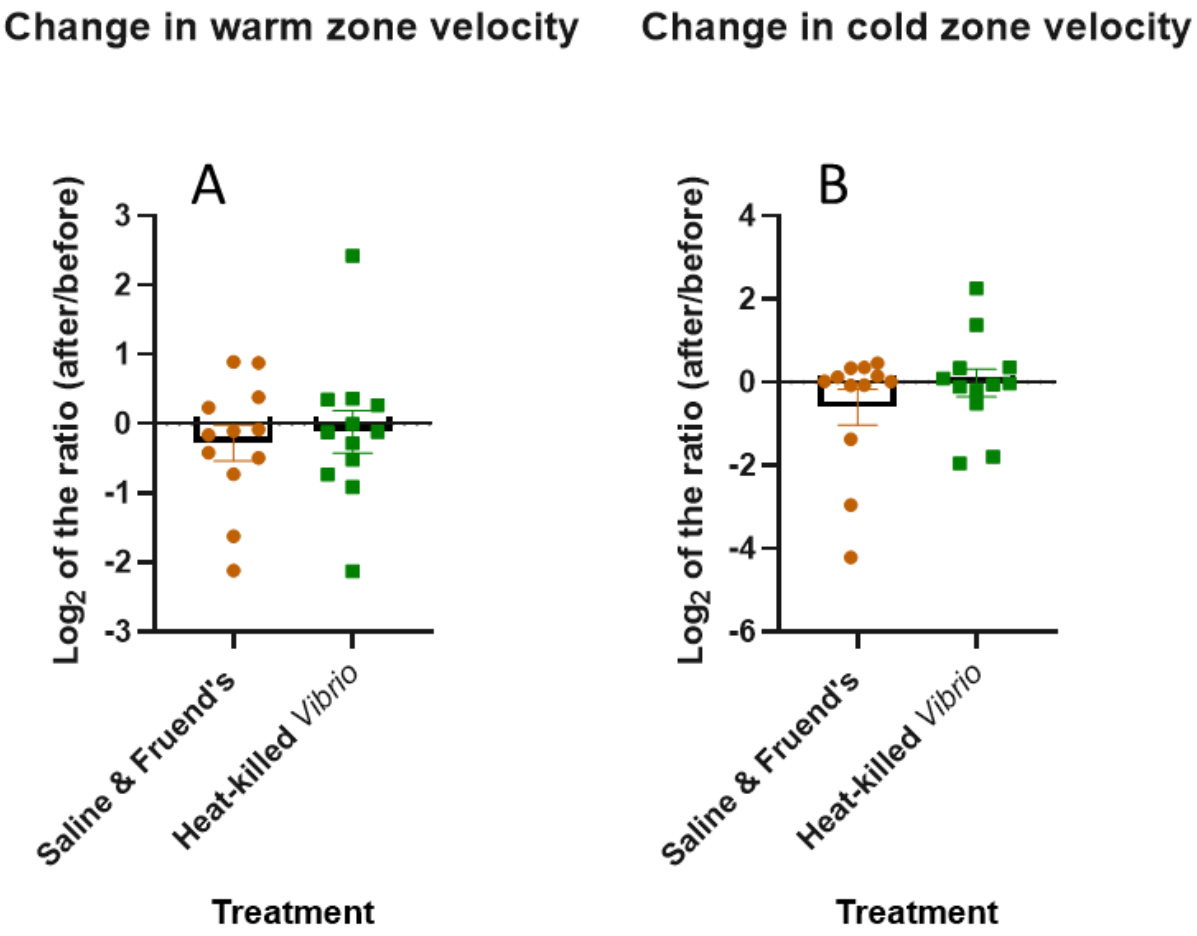
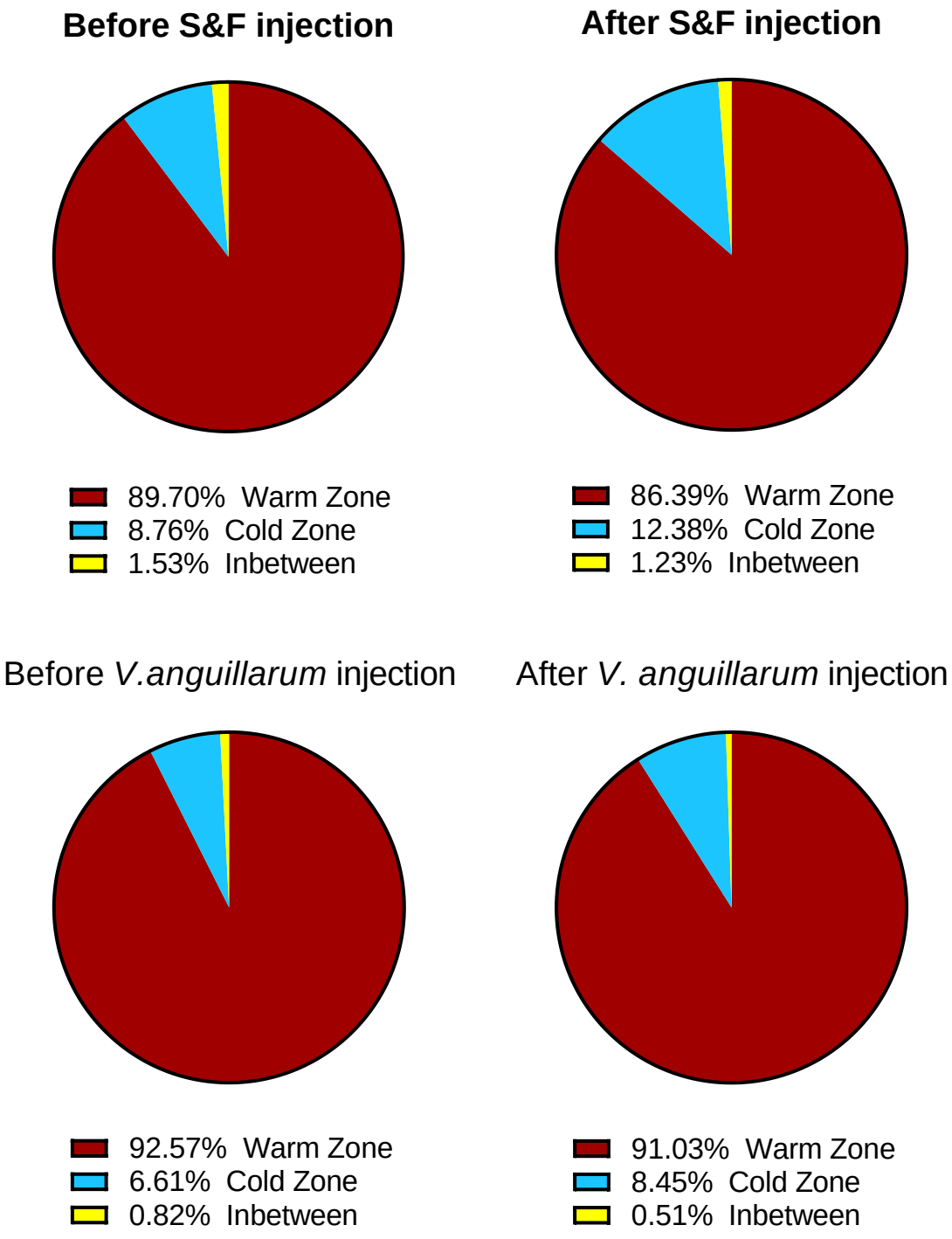


Figure 2.7: Fold change in the velocity before and after injection for both treatments in the different sides of the tank. Mean value of the log fold change of the velocity pre and post injection in the warm zone (A) or the cold zone (B). No significant differences were found between the treatments using an unpaired T-test (A) and the Mann-Whitney test (B) (n=12, α=0.05).

Overall the fish spent most of the time on the warmer side of the tank (25°C). The fish that were injected with saline and Freund's incomplete adjuvant spent 89.7% of the day before injection and 86.4% of the day after injection on the warm side (Fig 2.8). The fish that were injected with heat-killed *V. anguillarum* spent 92.6% of the day on the warmer side of the tank before injection and 91% of the day on the warmer side after injection (Fig 2.8). The amount of time spent on each side of the choice tank was compared using a Mann-Whitney test for the warm side and an unpaired t-test for the cold side. To reduce some of the individual variability, the time spent on the warm or cold side after injection was divided by the amount of time spent on the warm/cold side before injection for each fish. For the saline and Freund's incomplete adjuvant group the $\log_2$ of the fold change for the warm side was -0.0504 ± 0.0791 and 0.0108 ± 0.4936 for the cold side. For the group that was injected with heat-killed *V. anguillarum* the fold change in time was -0.0642 ± 0.0959 for the warm zone and -0.5581 ± 0.4814 for the cold zone (Fig 2.9a and 2.9b). There was no difference found between the change of time spent on the warmer side or the colder side between the two treatments after injection (Fig 2.9a & 2.9b).



(n=12)

Figure 2.8: Mean percent of the day that was spent in each of the zones during the behavioural study before and after injection for both treatments.

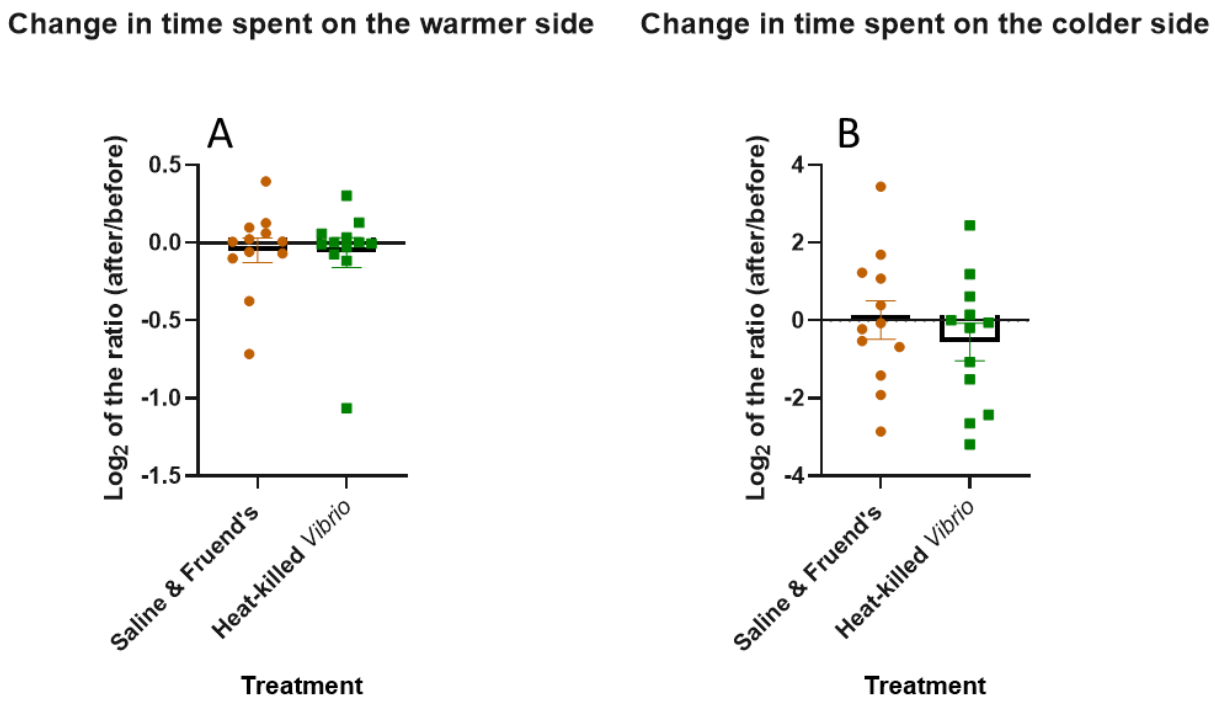
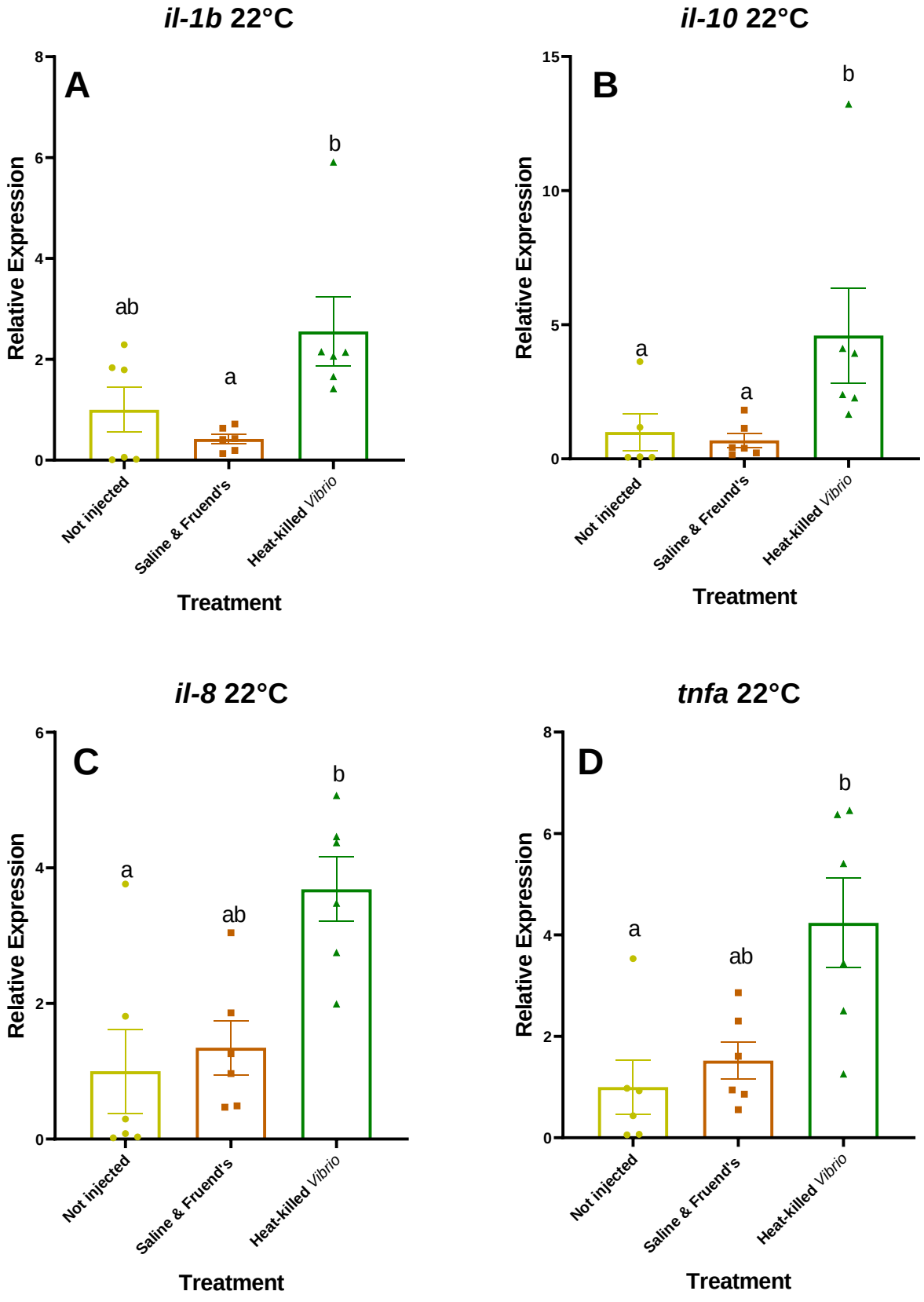


Figure 2.9: Fold change of the amount of time spent on the warmer and colder sides for both treatments. Mean of the log fold change of the time spent in the warm (A) or the cold zone (B) after injection divided by the time spent in either zone before injection for both treatments. No significant differences were found between the treatments on the warm side (A) using a Mann-Whitney test and no significant differences were found using an unpaired T-test for the cold side (B) (n=12).

3.4 RT-qPCR

At 22°C the RT-qPCR analysis showed more differences than at 27.5°C. The general trend for the RT-qPCR at 22°C was that the heat-killed *V. anguillarum* treatment group was significantly elevated compared to either the non-injected control group or the saline and Freund's injected group or both. The RT-qPCR targets that had significant differences between the treatment groups at 22°C were *il-1b*, *il-10*, *il-8* and *tnfa* **(**Fig 2.10). A one-way ANOVA or Kruskal Wallis test was used to analyze the differences between the ΔΔCQ values. Tukey's post hoc test was then used to see where the differences were between the groups. The targets where no significant differences were seen between any of the treatments were *ampka1* and *nf-kb* (Fig 2.11).


il-1b 22°C
A
Relative Expression
8
6
4
2
0
ab
a
b
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment
il-10 22°C
B
Relative Expression
15
10
5
0
a
a
b
Not injected
Saline & Freund's
Heat-killed Vibrio
Treatment
il-8 22°C
C
Relative Expression
6
4
2
0
a
ab
b
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment
tnfa 22°C
D
Relative Expression
8
6
4
2
0
a
ab
b
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment

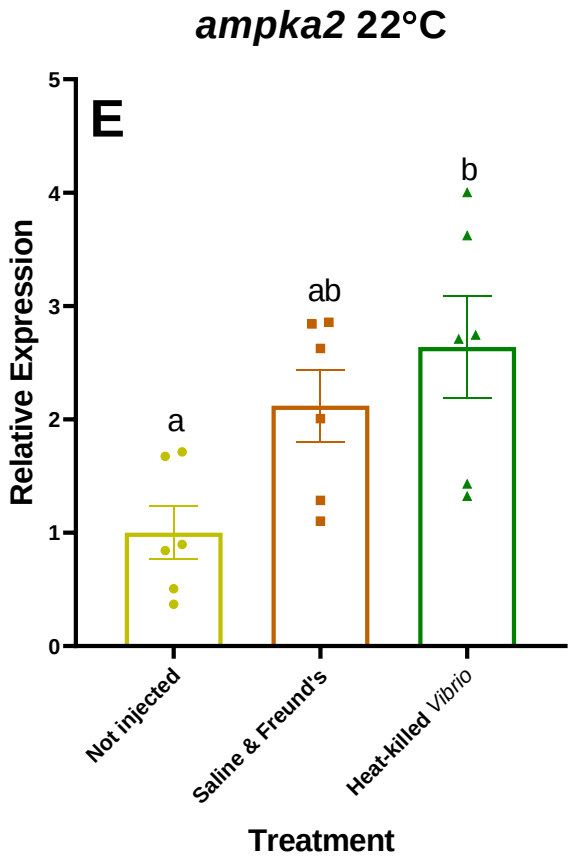


Figure 2.10: Relative expression in the spleen 27hpi for mRNA transcripts with significant differences at 22°C. The transcripts are **A**) *il-1b*, **B**) *il-10*, **C**) *il-8*, **D**) *tnfa*, and **E**) *ampka1* for spleen tissue of fish acclimated and experimented at 22°C. If two columns do not share any letters than they are significantly different from each other (α=0.05). A Kruskal-Wallis test was used for determining significant differences with Dunn's post hoc test for **A**, **B**, **C**, and **D**. A one-way ANOVA was used with Tukey's post hoc test for **E** (n=5/6).

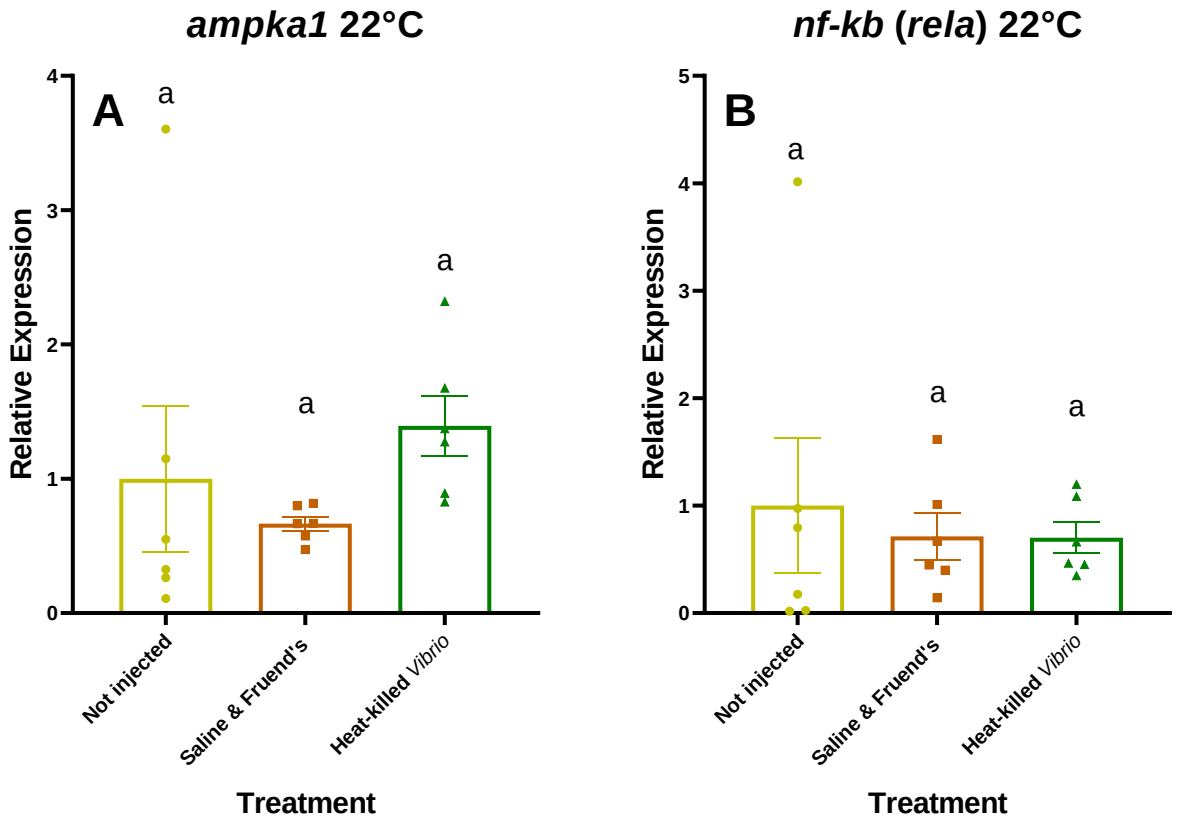


Figure 2.11: Relative expression in the spleen 27 hpi for mRNA transcripts with no significant differences at 22°C. The transcripts are **A**) *ampka1* and **B**) *nf-kb* transcript abundance from spleen tissues from fish acclimated to 22°C. No significant differences were found using a Kruskal-Wallis test (n=6).

At 27.5°C only one gene target (*tnfa*) demonstrated a significant difference in transcript levels between the treatment groups (Fig 2.12). This difference was between the non-injected control group and the Heat-killed *V. anguillarum* treatment group and it was in the opposite direction (significant decrease) of the difference that was seen at 22°C where there was a significant increase in the *tnfa* expression for the heat-killed *V. anguillarum* treatment group compared to the non-injected group. There was found to be no significant differences in the relative transcript abundance of; *il-1b*, *il-10*, *il-8*, *ampka1*, and *nf-kb* (Fig 2.13). A one-way ANOVA or Kruskal-Wallis test was used to look for differences between the treatment groups.

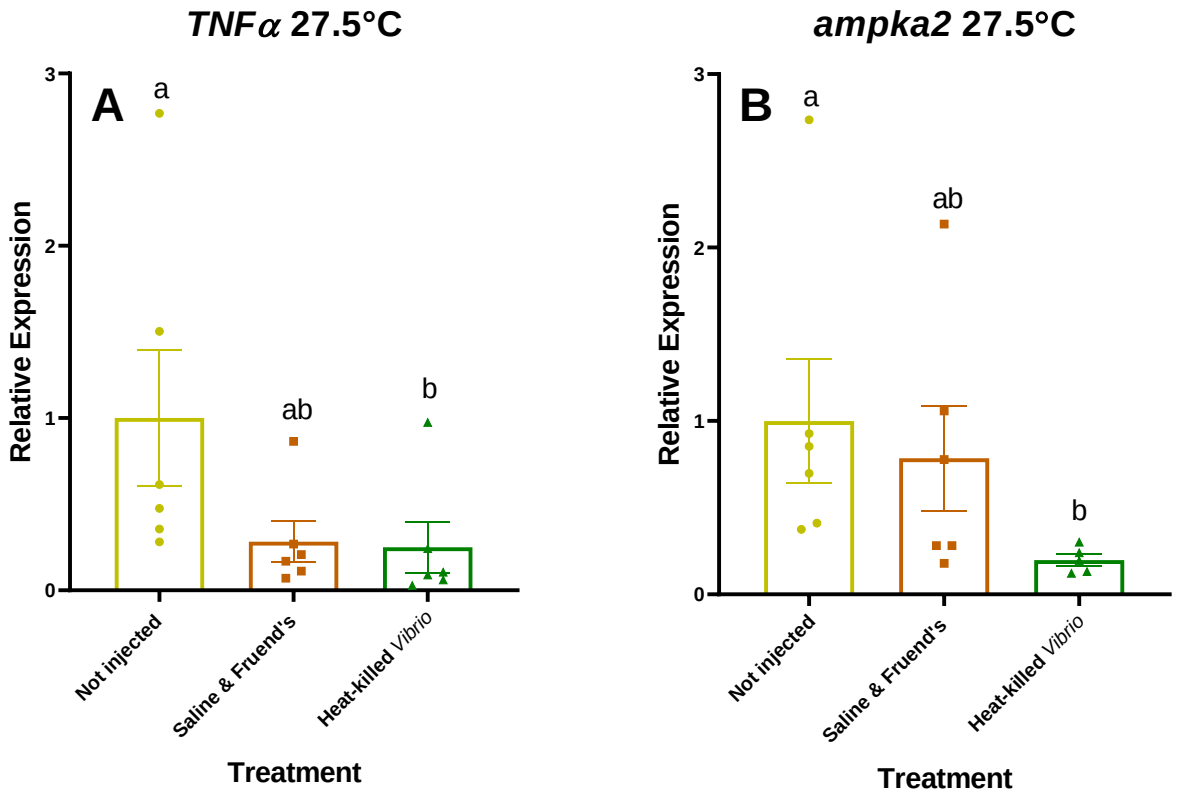
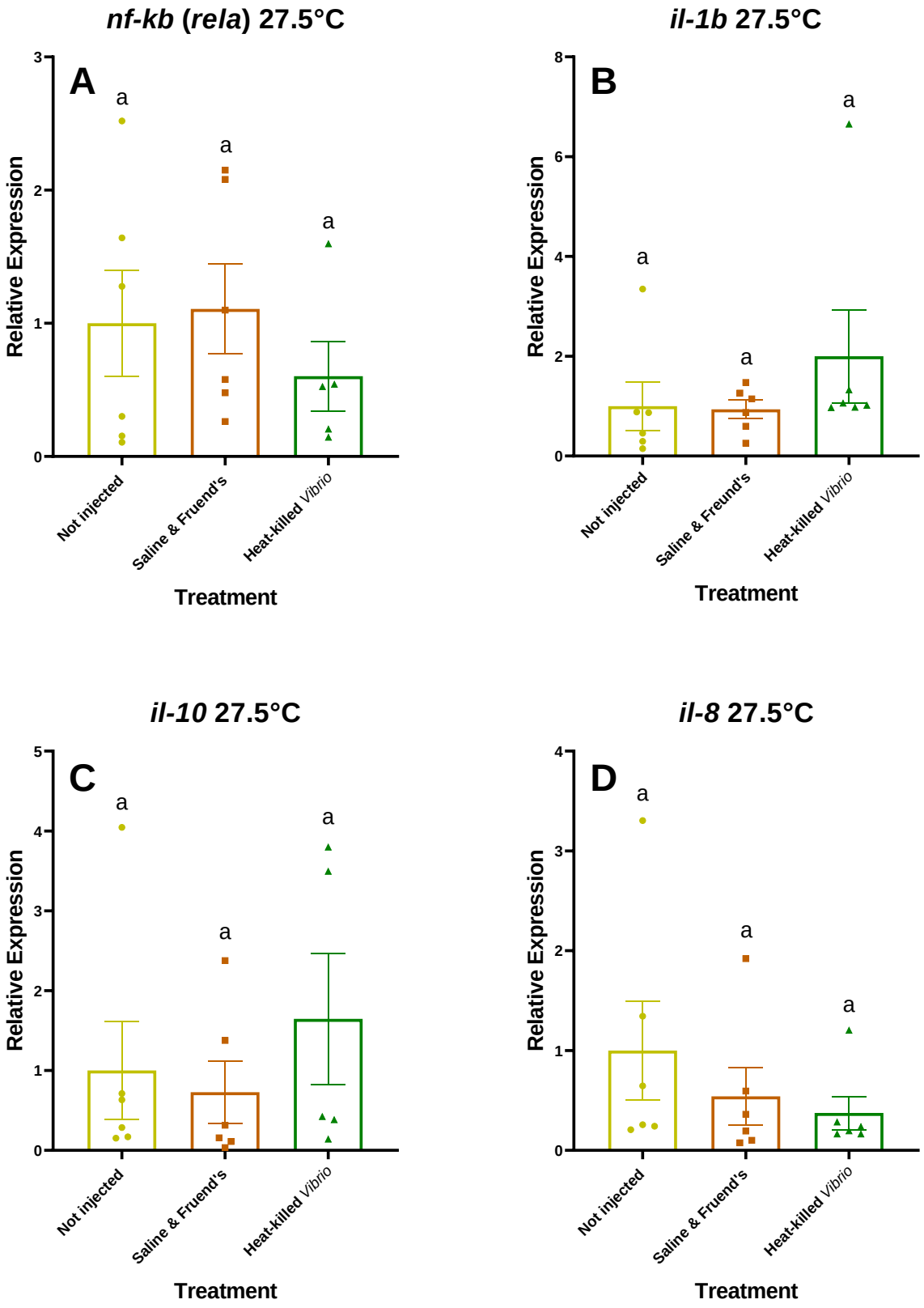


Figure 2.12: Relative expression in the spleen 27 hpi for mRNA transcripts with significant differences at 27.5°C. The transcripts are **A**) *tnfa* and **B**) *ampka2* for zebrafish that were acclimated to 27.5°C. A significant difference was found between the non-injected group and the heat-killed V. anguillarum treatment group for both targets. Differences were tested using the Kruskal-Wallis test and Dunn's post hoc test (n=6).


nf-kb (rela) 27.5°C
A
a
a
a
Relative Expression
0
1
2
3
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment
il-1b 27.5°C
B
a
a
a
Relative Expression
0
2
4
6
8
Not injected
Saline & Freund's
Heat-killed Vibrio
Treatment
il-10 27.5°C
C
a
a
a
Relative Expression
0
1
2
3
4
5
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment
il-8 27.5°C
D
a
a
a
Relative Expression
0
1
2
3
4
Not injected
Saline & Fruend's
Heat-killed Vibrio
Treatment

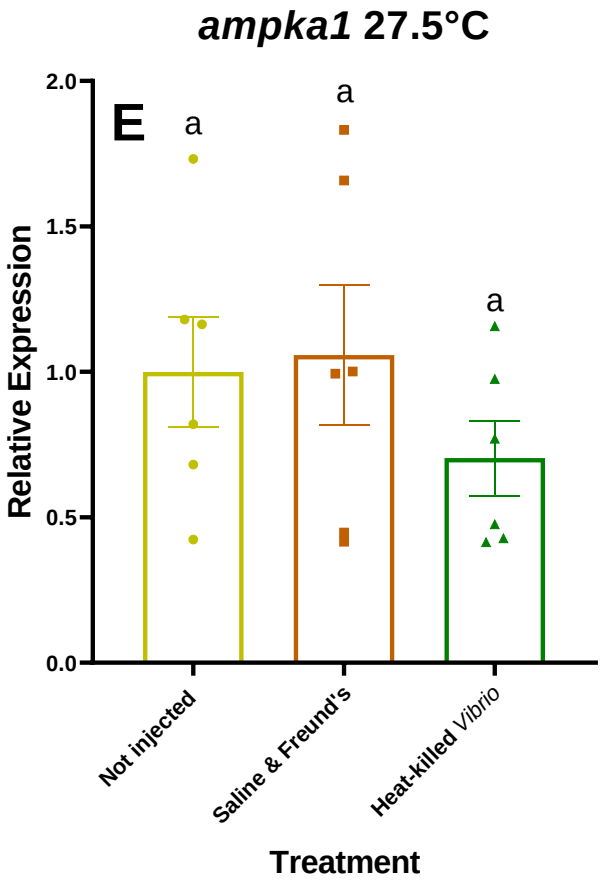


Figure 2.13: Relative expression in the spleen for mRNA transcripts with no significant differences at 27.5°C. The transcripts are **A**) *nf-kb* **B**) *il-1b*, **C**) *il-10*, **D**) *il-8*, and **E**) *ampka1*. The tissues were spleen from zebrafish acclimated to and experimented at 27.5°C. No significant differences were found using the Kruskal-Wallis test (**B**, **C**, **D**) or a standard one-way ANOVA (**A** & **E**) (n=5/6).

Chapter 4: Discussion

4.1: Aim of the study

This study aimed to better understand energetic demands of the immune response in a model organism, the zebrafish. Zebrafish studies have tried to understand the impacts that different stressors and environmental pollutants have on the energetics of zebrafish, but no studies have examined the energetics of the immune response [50-52]. Additionally, the zebrafish immune system has been extensively studied as well[25]. These two factors together explain why zebrafish are an excellent organism to study the interactions of the immune response on energetics because both its metabolic and immune pathways are very well understood. The first target of this study was to identify energetic changes that come with the immune response. However, this also aimed to establish the behavioural changes in zebrafish due to the immune response. This included further expanding our knowledge of behavioural fever in zebrafish as well as measuring if the movement of the fish changed during immune stimulation. The movement of the fish was expected to decrease after injection with the immune stimulant heat-killed *V. anguillarum*, as there are well defined sickness behaviours including an increase in lethargy in mammals and other vertebrates[89].

4.2: Energy consumption due to the immune response

4.2.1: Increases in oxygen consumption rate

It was hypothesized that there would be an increase in the RMR of zebrafish after injection with heat-killed *V. anguillarum*, as it has been shown in mice, brandt voles, and tuco-tucos for there to be an increase in the oxygen consumption rate after induction of the immune response[79-81]. However, comparison between these studies is challenging, as this would be equating endothermic animals to an ectothermic poikilotherm. This difference is important

because the febrile response is a valuable part of the innate immune response. Endotherms are able to boost their temperature internally by increasing their metabolic rate, whereas ectotherms rely on the external environment for a source of heat, so any difference in the oxygen consumption rates of the fish will be strictly due to the activation of the zebrafish immune response.

In this study, we found that there was a significant increase in the oxygen consumption rate of zebrafish after injection with heat-killed *V. anguillarum* at both the 27.5°C treatment group and at the 22°C treatment group (Fig 2.3 & 2.4). This is the first time that an increase in oxygen consumption rate due to the immune response has been shown in fish. Two other studies have looked at the effect the immune response has on the routine metabolic rate of fish, however, these studies did not find any changes. The first study by Zanuzzo et al. 2015, injected with steelhead trout (*Oncorhynchus mykiss*) with formalin killed *Aeromonas salmonicida*[120]. They did not find any changes in the RMR of steelhead trout injected with formalin killed *A. salmonicida* compared to sham injected fish[120]. One potential reason for the difference could be that the zebrafish in this study had a greater response to the immune challenge because they may have been stressed. This can be shown in Figure 4.12 where the heat-killed *V. anguillarum* group had a significantly lower relative transcript abundance of *tnfa* compared to the non-injected group, implying the zebrafish were stressed. Additionally, the lights turning on and off in the aquatic facility increased the RMR of the zebrafish (Figures 2.2&2.4) which also implies the zebrafish became stressed at these times. The second study by Zhang et al. (2019) looked at the oxygen consumption rate of salmon infected with Piscine orthoreovirus (PRV) but they did not find any significant increase in oxygen consumption rate[121], which was attributed to virulence. Zhang et al. (2019) describe PRV as being found in almost every wild and captive salmon with limited

mortality. *V. anguillarum* on the other hand can be very virulent and can cause mortality making it a dangerous pathogen[39]. Potentially, salmon have become extremely tolerant to PRV because it is so widespread and does not cause mortality, although this remains to be investigated. The increase in oxygen consumption rate in zebrafish was a 29% increase at 27.5°C and a 20% increase at 22°C. At both temperatures the oxygen consumption rate was elevated for the heat-killed *V. anguillarum* compared to both the non-injected group and the group that was injected with just saline and Freund's incomplete adjuvant. The elevation in the *V. anguillarum* group implies that it is not just the inflammatory response associated with tissue repair from the injection that is causing the elevation in oxygen consumption rate but also the need of the organism to eliminate the pathogen that was injected into the organism. This is likely due to the production of cytokines and complement system proteins associated with innate immunity causing the increase in the oxygen consumption rate.

4.2.2: Temperature differences

Even though there was a significant increase in oxygen consumption rate at both temperatures (27.5 and 22°C) the patterns displayed are not the same. At 27.5°C, the oxygen consumption rate was elevated for the entire 24-hour measurement period (Fig 2.4). At 22°C though, the oxygen consumption rate did not become elevated until 15 hours post injection (Fig 2.3). A longer time to a measurable increase in oxygen consumption rate implies that temperature had an effect on the activation of the immune response to heat-killed *V. anguillarum* at 22°C. The colder temperature (22°C), which is below the thermal preference of zebrafish, was delayed compared to the time it took for there to be a measurable increase in the oxygen

consumption rate at 27.5°C. A delay in the immune response at colder temperatures agrees with the literature that the immune response in fish is impaired at colder temperatures[112,122].

There were also differences seen in the mRNA transcript expression between the two temperatures at 27 hours post injection. The main differences between the two temperature were that there were many significantly elevated cytokines between the *V. anguillarum* treatment group and the other treatments at 22°C but there were very few differences seen at 27.5°C and, if there were any differences, they were in the opposite direction compared to what was expected and also shown at 22°C. Cytokines were measured to validate that there was an immune response taking place to the heat-killed *V. anguillarum*. At 22°C the RT-qPCR demonstrated that there was a transcriptional increase in *il-10*, *il-8*, *il-1b*, and *tnfa*. These cytokines have been shown to be upregulated during infection in fish with LPS from *Salmonella typhimurium*[123]. Taking the results of the RT-qPCR analysis together, there is evidence that there is evidence of an 3-4 fold increase of cytokines for the heat-killed *V. anguillarum* treatment at 22°C. However, at 27.5°C there was very little evidence of an immune response to the heat-killed *V. anguillarum*. The only significant difference that was shown was between the heat-killed *V. anguillarum* group and the not injected group in the relative transcript abundance of *tnfa* after 27 hours post injection (Fig 2.12). Additionally, *tnfa* relative expression was significantly reduced in the heat-killed *V. anguillarum* group at 27.5°C, the opposite of what was seen at 22°C.

ampka2 relative expression was significantly elevated in the *V. anguillarum* treatment group compared to the not injected group at 22°C (Fig 2.10). An upregulation of *ampka2* transcript abundance implies that the cells in the spleen are more tightly regulating the expenditure of energy likely due to a decreased quantity of ATP compared to AMP[124]. This correlates with the respirometry data that showed at 27 hours post injection the heat-killed *V.*

anguillarum treatment group had a significantly increased oxygen consumption rate implying the production of more ATP through aerobic respiration. Since, the relative expression difference is only seen between the heat-killed *V. anguillarum* treatment group and the not injected group it means that the upregulation is not due solely to the presence of PAMPs but also due to the injection. When looking at the 27.5°C experiment, the opposite was seen. The heat-killed *V. anguillarum* treatment group had a significantly decreased relative expression abundance of the *ampka2* transcript abundance compared to the not injected group (Fig 2.12). Again, there was no difference between the heat-killed *V. anguillarum* treatment group and the saline and Freund's treatment group. This indicates that the differences that are observed between the not injected group and the heat-killed *V. anguillarum* treatment group are due to a combination of both the injection and the presence of the PAMPs.

The inverse relationship between the two temperatures for *ampka2* and *tnfa* relative expression can potentially be explained by the amount of time post injection, since an increase in the oxygen consumption rate was observed at both temperatures. At 27.5°C, the oxygen consumption rate was increased for the full 24-hour measurement period (Fig 2.4). For the 22°C experiment the oxygen consumption rate was elevated for 12 hours prior to sampling (Fig 2.3). A longer time before there was a measurable increase in the oxygen consumption rate at 22°C implies that the response to injection of heat-killed *V. anguillarum* was slower at 22°C compared to 27.5°C. The transcript response for cytokines could be over by 27 hours post injection at 27.5°C. A faster response at higher temperatures would make sense as it has been shown in rainbow trout hyperdermal fibroblast cells that reduced temperatures increase the amount of time required for an immune response[122]. A faster response time at warmer temperatures can explain why there is very limited evidence of an immune response at 27.5°C. Since cytokines need to be

tightly regulated production can be rapidly activated and inactivated to prevent over production. Therefore, if production was turned off at 27hpi at 27.5°C significant differences would not be expected between treatments and if there were changes it would be an expected down regulation in the treatment that just finished an immune response. Stockhammer et al. (2009) found that by 24 hours post injection LPS from *Salmonella typhimurium* was cleared from embryonic zebrafish demonstrating that heat killed bacteria can be cleared from the zebrafish rapidly[123].

4.3: Behaviour changes

4.3.1: Behavioural fever

As stated previously, ectotherms have been shown to display a behavioural fever when presented with an immune challenge[96,97]. Behavioural fever has also been specifically shown in zebrafish in response to double stranded RNA[95]. In a study by Boltaña et al. (2013) zebrafish were injected intraperitoneally with double stranded RNA. Zebrafish were then placed into a choice tank where they could choose a temperature they preferred, and it was seen that there were significantly more fish found on average in the chamber just warmer (33°C) than the thermal preference chamber (29°C) after injection with double stranded RNA compared to the number of sham injected fish (Boltaña et al. 2013). With the evidence of behavioural fever existing in zebrafish, the main priority of this study was not to demonstrate that zebrafish exhibit a behavioural fever but to calculate the additional energy required for an immune response in zebrafish, including the additional energy that would be required from spending more time in warmer water during a behavioural fever. The secondary objective was demonstrating a behavioural fever as part of the anti-bacterial immune response in zebrafish. Calculating the amount of extra time spent in the warmer was not ideal using the data from Boltaña et al. (2013)

because they did not track individual fish. In the experimental setup for this thesis it was possible to track the individual fish to see how much extra time a fish injected with heat-killed *V. anguillarum* spent in the warmer chamber compared to the control injected fish. Using the amount of extra time spent in warmer water over the course of a day it would be possible to calculate the additional energy required for a behavioural fever. This could be calculated using the following equations:

Equation 1:

$$\frac{(RMR_L P_L) + \{[RMR_L(1+b)^{\Delta C}] \times P_E\} - RMR_L}{RMR_L} \times 100\%$$

After dividing out RMR_L from equation 1, a simplified equation (equation 2) is present without the need to know the oxygen consumption rates. What does need to be known though is amount of time spent in the lower (preferred) water temperature and the additional time spent in warmer water after induction of an immune response.

Equation 2:

$$P_L + [(1+b)^{\Delta C} \times P_E] - 1 \times 100\%$$

If the b value is not known for the species of fish but you know the RMR of the fish at both the lower water temperature and the elevated water temperature, the increase in oxygen consumption rate attributed to the behavioural fever can also be calculated using equation 3.

Equation 3:

$$\frac{[(RMR_L P_L) + (RMR_E P_E)] - RMR_L}{RMR_L} \times 100\%$$

In these equations:
RMR_L = RMR at the lower water temperature
P_L = Percent of the day spent at the lower water temperature
b = The fish specific percent increase in oxygen consumption rate per degree Celsius
ΔC = The difference in temperature between the preferred and elevated water temperatures
P_E = Percent of the day spent at the elevated water temperature
RMR_E = $[RMR_L(1+b)^{\Delta C}]$ (From equation 1) = RMR at the elevated water temperature

However, in this study no behavioural fever was demonstrated (Fig 2.8 & 2.9). Even though no behavioural fever was demonstrated in this study it does not mean that zebrafish do not demonstrate a behavioural fever in response to bacterial PAMPs. Likely, the fact that no behavioural fever was demonstrated was because the experiment was conducted at 22.5°C. We chose to perform the experiment at 22.5°C because there was a transcriptional increase of cytokines for the heat-killed *V. anguiollarum* after 27 hours post-injection. A transcriptional response happening 27 hours post-injection allowed us to acclimate the zebrafish overnight to the choice tank and then monitor the behaviour of the fish the following day. As previously mentioned, the thermal preference for zebrafish is 28.5°C. Therefore, 22.5°C is below the thermal preference of zebrafish. For this reason, the zebrafish were acclimated to 22°C water for four months prior to the experiment. However, the zebrafish moved straight for the warmer water and spent approximately 90% of the day in the warmer side of the choice tank (25°C) before and after injection for both the saline and Freund's treatment and the heat-killed *V. anguillarum* treatment (Fig 2.8). Since, the zebrafish spent the majority of the time in the warmer water regardless of treatment, it masked the possibility of seeing a behavioural fever.

Making some assumptions from the data from Boltaña et al. (2013) and combining it with some of the data from this study, it would be possible to estimate the increase in RMR due to behavioural fever from injection with double stranded RNA in zebrafish. If we assume that all fish spent an equal amount of time in the 33°C chamber of the choice tank in the Boltaña et al.

study, we know that on average there were approximately 1.8 more fish in the 33°C chamber at any given time after injection with double stranded RNA compared to the sham injected fish. Since each group has 10 fish, 1.8 more fish at any given time works out to each fish spending about 18% more time in the warmer zone after injection with double stranded RNA. Therefore, 18% will be the P_E value for equation 1. Since the percent total of the day needs to be equal 100, 82% will be the P_L value. The authors report that the average temperature difference between chamber 5 and chamber 4 was 3 ± 0.5°C, making the ΔC value 3[95]. To calculate the b value for zebrafish we rearrange the equation $RMR_E = RMR_L(1+b)^{\Delta C}$ to get; $b = \sqrt[\Delta C]{\frac{O_E}{O_P}} - 1$. Using the RMR of the not injected zebrafish group at 22°C (0.2692mgO_2/g/hr) and 27.5°C (0.402 mgO_2/g/hr) from this thesis for RMR_L and RMR_E respectively and the difference between the two temperatures (5.5°C) as ΔC, we find that the value of b for zebrafish is 0.076 or 7.6% increase in RMR per degree Celsius.

With all the values now known for equation 2, it can be filled in to get:

$$0.82 + [(1 + 0.076)^3 \times 0.18] - 1 \times 100\% = 4.4\%$$

Therefore, based on the additional extra time that zebrafish chose to spend in the warmer chamber after injection with double stranded RNA compared to sham injected zebrafish in Boltaña et al. (2013). And using the specific increase in RMR due to temperature per degree Celsius in this study, it can be estimated that the behavioural fever in zebrafish for double stranded RNA results in a 4.4% increase in the RMR of the zebrafish

4.3.2: Locomotion

Since, a characteristic sickness behaviour in mammals and other vertebrates is a reduction in movement[89], it was hypothesized that zebrafish presented with an immune challenge

would decrease the amount that they moved to conserve energy. To test this hypothesis the distance the zebrafish moved before and after injection with either saline and Freund's incomplete adjuvant or with heat-killed *V. anguillarum* in saline and Freund's incomplete adjuvant was measured. After examination of the distance moved before and after injection it was found that there were no significant changes in the absolute distance that the fish moved in either treatment (Fig 2.5). To reduce the variability of the data for the distance each fish moved before and after injection the fold change in distance moved after injection compared to before injection was calculated. Again, there was found to be no significant difference in the fold change in either of the treatment groups at 22.5°C (Fig, 2.6). The last metric that was tested to see if there was a change in the movement patterns of zebrafish after injection with heat-killed *V. anguillarum*, was the change in velocity in the warm or the cold zones. This metric was calculated because it was thought that even though the amount of time spent in the warmer side compared to the colder side did not change, and the total distance travelled did not change, the combination of the two factors may have changed. Perhaps the fish moved less in the warmer side and more in the colder side leading to a net change of zero in the distance moved. When the change in velocity was analyzed for both the warmer and the colder side of the choice tank, there was found to be no significant differences (Fig 2.7).

With all of the data from the absolute distance moved, the fold change in the distance moved and the change in velocity in both the warm and the cold zones there was no change. Looking at the data as a whole, this experiment demonstrated that zebrafish acclimated to 22°C did not demonstrate any changes in movement when presented with an immune challenge of heat-killed *V. anguillarum*. This result could likely be due to the fact that a reduction of movement is less beneficial for an ectotherm compared to an endotherm, as a fish needs to

search out and find warmer water in order to elevate their body temperature for behavioural fever. Since, behavioural fever has already been demonstrated to increase survival in fish[95,107,110], it is likely that the trait of moving during an immune response was selected for to allow fish to regulate their body temperatures and increase survival rate through movement during an immune response.

4.4: Future directions

4.4.1: Expansion of the respirometry experiments

The respirometry experiments showed clear evidence that the RMR of fish injected with heat-killed *V. anguillarum* increased. However, at 27.5°C there was no evidence with the relative transcript abundance of cytokines for an immune response in the zebrafish. This is believed to be because the transcript response had already occurred prior to 27 hours post injection when the tissue samples were collected. For this reason, a logical next step would be to inject zebrafish that are acclimated and held at 27.5°C with heat-killed *V. anguillarum* and then collect tissues at an earlier time point such as 8 hours post injection and measure the relative transcript expression of pro-inflammatory cytokines such as *tnfa* and *il-1b* then to ensure that an immune response is taking place. A transcript response should be seen at 8 hours post injection because in zebrafish acclimated to 28°C that were exposed to LPS of *Salmonella typhimurium* demonstrated an upregulation of *tnfa*, *il-8* and *il-1b* at 8 hours post injection[123]. Demonstrating a transcriptional response of cytokines would further show that the increase in RMR is due to the immune response.

Additionally, the respirometry experiment could be expanded with the use of a live pathogen. The use of a live pathogen would provide data about a more ecologically relevant

immune response, because in the wild and in aquaculture the pathogens are replicating within the host. This would provide a more accurate value for the energy expenditures it takes for a host to fight off a pathogen. Also, live pathogen challenge could allow for a way to measure the adaptive immune response and not just the innate immune response. Since, a live pathogen is replicating and can persist longer it would be possible to have the fish infected long enough to see the adaptive response. The energy requirements between the adaptive and innate immune responses could be compared to see if one is more costly than the other, and it is predicted that the adaptive immune response would lead to a larger increase in the RMR of the fish, as this response represents an up regulation in transcription, translation, and post-translational modification machinery. Further, as the innate response is constitutively expressed[27], the difference in scale between elevating the innate immune response levels from baseline to an induced state would be less of a change compared to inducing the adaptive response from very low levels to a highly active state.

4.4.2: Expansion of the behavioural study

The next step for the behavioural study would be to repeat the experiment at a higher temperature. The lower temperature would be the thermal preference for zebrafish (28.5°C) and the warmer side of the choice tank around 31.5°C. This would allow for a behavioural fever to be shown due to injection with heat-killed *V. anguillarum*. With a behavioural fever demonstrated a more accurate estimate of the additional energy demands of the immune response could be created. This estimate would combine both the increase in energy demands shown due to the induction of the innate immune response from the respirometry as well as the calculation of the additional energy that is required for spending longer durations in warmer water for the fish.

4.5: General conclusions

The predictions of this study were that there would be an increase in the RMR, zebrafish would demonstrate a behavioural fever, and zebrafish would have reduced movement after injection with heat-killed *V. anguillarum*. This study demonstrated the first part of the prediction to be true. Zebrafish injected with heat-killed *V. anguillarum* demonstrated an increased RMR compared to both the control groups at 22°C and at 27.5°C. The second prediction of zebrafish demonstrating behavioural fever in response to the heat-killed *V. anguillarum* was not demonstrated in this study. However, the study did not show evidence against a behavioural fever due to the zebrafish always moving to the warmer side of the tank independent of treatment. The final prediction that zebrafish would demonstrate increased lethargy after injection with heat-killed *V. anguillarum* was found to be incorrect. The data from this experiment showed that zebrafish did not appear to alter their movement patterns in response to the injection. So, this study demonstrates that zebrafish do not reduce their movement in response to injection with heat-killed *V. anguillarum*.

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Appendix

Table A.1: Significant differences across the time points for the oxygen consumption rate measurements at 22.5°C.

A 2-way ANOVA was used to find any significant differences (α=0.05)

Hours Post Injection	Hours it is different than
3	10.5, 22.5
3.5	10.5, 22.5
4	10.5
4.5	
5	10.5, 22.5
5.5	10.5, 22.5
6	10.5, 22.5
6.5	10.5, 22.5
7	10.5, 13.5, 22.5
7.5	10.5, 22.5
8	10.5, 22.5
8.5	10.5, 22.5
9	10.5, 22.5
9.5	10.5, 22.5
10	10.5, 22.5
10.5	3, 3.5, 4, 5-10, 12, 13-16, 18.5-19.5, 20.5-21.5, 24
11	13.5
11.5	22.5
12	10.5, 22.5
12.5	22.5
13	10.5, 22.5
13.5	7, 10.5, 11, 22.5, 23, 23.5
14	10.5, 22.5
14.5	10.5, 22.5
15	10.5, 22.5
15.5	10.5, 22.5
16	10.5, 22.5
16.5	22.5
17	
17.5	
18	22.5
18.5	10.5, 20.5, 22.5, 23.5, 24.5
19	10.5, 22.5
19.5	10.5, 22.5
20	22.5
20.5	10.5, 18.5, 22.5
21	10.5, 22.5
21.5	10.5, 22.5
22	22.5

22.5	3, 3.5, 5-10, 11.5-16.5, 18-22, 23-26
23	13.5, 22.5
23.5	13.5, 18.5, 22.5
24	10.5, 22.5
24.5	18.5, 22.5
25	22.5
25.5	22.5
26	22.5

Table A.2: Significant differences across the different time points at 22°C when only analyzing 15-27 hours post injection.
A 2-way ANOVA was used to analyze the differences (α=0.05).

Hours Post Injection	Hours it is different than
15	22.5
15.5	22.5
16	17, 22.5, 23.5
16.5	22.5
17	16, 22.5
17.5	22.5
18	22.5
18.5	20.5, 22.5, 23.5-24.5, 25.5
19	22.5
19.5	22.5
20	22.5
20.5	18.5, 22.5
21	22.5, 23
21.5	22.5
22	22.5
22.5	15-22, 23-26
23	21, 22.5
23.5	16, 18.5, 22.5
24	18.5, 22.5
24.5	18.5, 22.5
25	22.5
25.5	18.5, 22.5
26	22.5

Table A.3: Significant differences across the time points for the oxygen consumption rate measurements at 22.5°C.
A 2-way ANOVA was used to identify if there were differences and Tukey's post-hoc test was used to see where the specific differences were seen (α=0.05).

Hours Post Injection	Hours it is different than
3	
3.5	
4	14.5
4.5	
5	22
5.5	22
6	22
6.5	22
7	22
7.5	22, 22.5
8	22
8.5	22, 22.5, 23
9	10, 22
9.5	
10	9
10.5	
11	
11.5	
12	22, 22.5, 24.5
12.5	22, 24.5
13	22, 22.5, 23.5-24.5, 25.5
13.5	22, 22.5, 23.5
14	22, 23.5, 24.5, 25.5
14.5	4, 22, 22.5, 23.5-24.5, 25.5
15	22, 24.5, 25.5
15.5	22, 22.5, 23.5-24.5, 25.5
16	22, 22.5, 24, 24.5, 25.5
16.5	22, 22.5, 24, 24.5, 25.5
17	22, 22.5, 24.5
17.5	22, 24.5, 25.5
18	22, 22.5, 24, 24.5, 25.5
18.5	22, 22.5, 23.5, 24.5, 25.5
19	22, 22.5, 23.5-24.5, 25.5
19.5	22, 24.5
20	22, 22.5, 23.5-24.5, 25.5
20.5	22
21	22
21.5	
22	5-9, 12-21, 25, 26

22.5	7.5, 8.5, 12, 13, 13.5, 14.5, 15.5-17, 18-19, 30
23	8.5
23.5	13-14.5, 15.5, 18.5, 19, 20
24	13, 14.5, 15.5-16.5, 18, 19, 20, 25
24.5	12-13, 14-20
25	22, 24
25.5	13, 14-16.5, 17.5-19, 20, 26
26	22, 25.5